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Novel insight into the liver-lung axis in alcohol-enhanced acute lung injury.

Lauren G. Poole
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NOVEL INSIGHT INTO THE LIVER-LUNG AXIS IN
ALCOHOL-ENHANCED ACUTE LUNG INJURY

By

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B.S. University of Louisville, 2013

M.S. University of Louisville, 2015

A Dissertation

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DEDICATION

This dissertation is dedicated to my parents

Janet Weiss

And

Michael Poole

And to my husband

David Hardy

for their constant support and encouragement

In all my endeavors

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First and foremost, I would like to thank my mentor, Dr. Gavin Arteel, for his guidance and support, and for always encouraging me to pursue novel and challenging scientific questions. I would also like to thank my committee, including Dr. Jesse Roman for his expertise in alcohol-related lung injury and clinical insight, Dr. Gary Hoyle for his expertise in acute lung injury, and Drs. Leah Siskind and Christopher States for their expertise in mechanistic toxicology. I would also like to thank the Roman group, especially Dr. Edilson Torres-González for his training in surgical techniques and Jeffrey Ritzenthaler for his technical guidance, as well as Connie Schleuter in the Hoyle group for her assistance with pulmonary function measurements. Thank you to Dr. Jill Steinbach and her laboratory for providing not only the tamoxifen-loaded PLGA nanoparticles used in these studies, but also for their expertise with the guidance provided for working with these particles. Finally, the *in-vivo* studies presented in this dissertation would not have been possible without the constant guidance and assistance from Dr. Juliane Beier.

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ABSTRACT

NOVEL INSIGHT INTO THE LIVER-LUNG AXIS IN ALCOHOL-ENHANCED ACUTE LUNG INJURY

Lauren G. Poole

June 8, 2017

Background. Individuals who chronically abuse alcohol are almost 4 times more likely to develop Acute Respiratory Distress Syndrome (ARDS), the most severe form of Acute Lung Injury (ALI), but the mechanisms by which alcohol abuse sensitizes the lung to injury are poorly understood. However, the lung appears to share many parallel mechanisms of injury with the liver- a primary target of alcohol abuse. The overarching goal of this dissertation was therefore to expand on established mechanisms of alcohol-induced liver injury to ask innovative questions about mechanisms of alcohol-enhanced acute lung injury, as well as to develop new tools that may be used to gain novel insight into the liver-lung axis of alcohol-induced injury. **Methods.** Male mice were exposed to ethanol containing liquid diet either chronically (6 weeks) or in a chronic + binge pattern. Some mice were administered lipopolysaccharide (LPS) to induce acute lung injury. Lung injury and inflammation were assessed. To develop an animal model by which liver-lung interactions could be investigated, tamoxifen-loaded polymer nanoparticles were administered intrasplenically to a tamoxifen-inducible, Cre-mediated, dual-

fluorescent reporter construct. **Results.** Chapter III of this dissertation describes a mechanism by which plasminogen activator inhibitor-1 (PAI-1) is involved in alcohol-enhanced acute lung injury. Specifically, it was proposed that PAI-1-mediated fibrin accumulation promotes the aggregation of platelets, thereby propagating lung injury and inflammation. Chapter IV of this dissertation characterizes a recently-developed animal model of chronic + binge alcohol exposure, finding that animals exposed to chronic + binge alcohol exposure exhibit pulmonary inflammation and airway hyperresponsiveness. Finally, Chapter V of this dissertation develops an animal model to investigate liver-lung interactions during chronic alcohol exposure. It was found that tamoxifen-loaded polymer nanoparticles, when administered intrasplenically, selectively alter the genetics of hepatic cells, while avoiding other tissues, including the lung. **Discussion.** The work presented in this dissertation has, in conclusion, uncovered novel mechanisms by which alcohol sensitizes the lung to a second injury, shown that ethanol alone is sufficient to cause lung inflammation, and developed a novel animal method to examine liver-lung interactions during alcohol exposure.

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CHAPTER I

INTRODUCTION¹

A. **Background and rationale for this study**

1. Alcohol use and its impact

The discovery of fermented beverages was likely accidental and derived from improper food storage. As agrarian culture developed throughout the world, so did the intentional cultivation of crops for alcoholic beverage production. Alcoholic beverages were valued in ancient cultures for several reasons beyond them being a 'social lubricant' (1). In a time when potable water was difficult to acquire, alcohol acted as a relatively safe source of hydration. Additionally, alcohol's modest nutritional value supplemented malnourishment. Furthermore, alcohol had significant medicinal value as an antimicrobial agent. Taken together, the pervasive nature of alcohol consumption throughout the world is unsurprising. Even in cultures that forbid alcohol consumption, the development of such taboos speaks to the fact that these peoples have been exposed to alcohol consumption.

There are many potential benefits of alcohol consumption, as discussed above. Despite these benefits, the idea of a need for moderation in alcohol consumption is as ubiquitous as the consumption of alcohol itself; indeed, almost

¹ Parts of this section appear in Poole LG and Arteel GE. *Biomed. Res. Int.* 2016;2016:3162670

every culture frowns upon public intoxication and alcohol abuse and/or dependence. For example, Aristotle strongly extols the virtue of temperance in his work, "The Nicomachean Ethics." In modern society, alcohol abuse has an even more significant impact. For example, there is, on average, more than one alcohol-related driving fatality every hour in the US (2).

In addition to these social consequences, alcohol abuse significantly impacts health. Alcohol requires relatively high concentrations to exert many of its toxic effects in organisms and is therefore, arguably, not an incredibly potent toxin. However, alcohol must be consumed in relatively high doses to cause any noticeable inebriating effects; the legal driving blood alcohol content (BAC) in most US States (0.08% w/v) translates to ~20 mM ethanol. Therefore, the sheer volume of alcohol which humans consume is enough to offset its low potency. In fact, one could argue that alcohol is the most common poison voluntarily consumed at toxic doses by the human population. In the United States alone, over 17 million adults suffer from an alcohol use disorder (AUD), a condition that the National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines as "a chronic relapsing brain disease characterized by compulsive alcohol use, loss of control over alcohol intake, and a negative emotional state when not using." Chronic alcohol consumption/abuse has been demonstrated to directly damage several organs, including liver (3), lung (4), skeletal muscle and heart (5), the brain (6), and the pancreas (7). Additionally, alcohol consumption increases the risk of developing several cancers; it is considered a group 1 carcinogen for cancers of the GI tract, liver, breast and pancreas by the International Agency for

Research on Cancer (8). Ultimately, alcohol consumption is responsible for ~6% of all disability-adjusted life years (DALY) lost in the United States (9), most of which are attributable to alcohol-induced toxicity as opposed to alcohol-related accidents.

2. Alcoholic Liver Disease

The liver is located between the intestinal tract and the rest of the body, making it a critical organ in the clearance of toxins and xenobiotics, including alcohol, that enter the portal blood. The concentration of alcohol found in the portal blood is much higher than those in the systemic circulation. Additionally, the liver is the primary site of alcohol metabolism, which produces many toxic metabolites. Therefore, it is unsurprising that the liver is a primary target of alcohol toxicity. Although excessive alcohol consumption has been associated with organ toxicity since ancient times, the first suggestion that alcohol consumption may directly cause organ damage is credited to Thomas Addison in 1836 (10).

Alcoholic Liver Disease (ALD) affects millions of patients worldwide each year. The progression of ALD is well-characterized and is actually a spectrum of liver diseases, ranging initially from simple steatosis, or fat accumulation, to inflammation and necrosis (often called steatohepatitis), and ultimately, to fibrosis and cirrhosis. Although the risk of developing ALD increases in a dose- and time-dependent manner with alcohol consumption (11, 12), only a small fraction of even the heaviest drinkers develop the severe form of the disease, suggesting

that other environmental (e.g., hepatitis B virus (HBV) or hepatitis C virus (HCV) infection) or genetic (e.g., gender or polymorphisms in key genes) factors contribute to overall risk (13). Clinical management of ALD primarily focuses on maintaining abstinence in the alcoholic, and on treating sequelae associated with acute alcoholic hepatitis or cirrhosis (14). The effects of decompensation (e.g., hepatorenal syndrome) usually lead to the death of the patient, except in the case of a successful liver transplant (15). Furthermore, the overall risk of developing hepatocellular carcinoma (HCC) increases roughly 20-fold by preexisting cirrhosis, even in patients in which compensation is maintained (*i.e.*, ‘stable cirrhotics’) (16). HCC has an even more dismal prognosis than cirrhosis with very high mortality rates (17).

3. Alcoholic lung phenotype

The lung is also recognized as a target of chronic alcohol abuse, and alcohol-related lung injury is estimated to account for tens of thousands of deaths in the United States each year (18). Although chronic alcohol consumption is not directly linked to the development of an “alcoholic lung disease” (*i.e.*, as it is to liver disease), alcohol is a significant risk factor in the morbidity/mortality of lung damage from other causes. For example, acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), occurs 3.7 times more often in individuals meeting the diagnostic criteria for alcohol use disorders (19, 20). Furthermore, Moss and colleagues demonstrated that chronic alcoholics who developed ARDS showed in-hospital mortality rates of 65%, whereas

nonalcoholic patients had mortality rates of 36% (19-21). The exact mechanisms by which alcohol mediates these effects on the lung are unknown, but experimental ethanol exposure has been associated with the up-regulation of pro-inflammatory cytokines (22-24), disruption of regulatory signaling pathways (25), activation of tissue remodeling (26, 27), and the induction of oxidative stress in rodent lungs (28). All of these factors may promote the development of the “alcoholic lung” phenotype. This phenotype appears to enhance the host’s susceptibility to serious lung diseases, including ARDS, ALI following severe trauma (e.g. car accident, gun shot, etc.), and respiratory infection (e.g., pneumonia) (18). In fact, having an alcoholic use disorder is a major independent risk factor for development and susceptibility to sepsis-induced ARDS, even after adjusting for smoking status and co-morbidities (21).

To investigate mechanisms by which alcohol exposure sensitizes the lung to sepsis-induced ARDS, experimental mouse models may be utilized. Intraperitoneal (i.p.) injection of bacterial lipopolysaccharide (LPS), or endotoxemia-induced lung injury, is one such animal model employed to study ALI and ARDS (29, 30). Indeed, this model has been demonstrated to cause sequestration of neutrophils in the pulmonary vasculature and interstitium (31, 32). Furthermore, i.p. LPS administration has been demonstrated to cause pulmonary edema, diffuse alveolar damage, and induction of inflammatory cytokines and chemokines, such as IL-6 and MCP-1 (33). Systemic administration of LPS (versus intratracheal) is a particularly relevant model to investigate mechanisms of alcohol-induced organ pathology, as chronic alcohol

consumption has been demonstrated to increase systemic LPS in patients (34). Systemic endotoxin may, in turn, damage the lung directly or via inflammatory mediators released from other tissues. Furthermore, although intratracheal administration of LPS has been demonstrated to cause a large influx of PMNs into the alveoli, there are concerns that this model may include confounding factors, such as aspiration injury (32). In the setting of chronic alcohol exposure, ethanol feeding enhances glutathione depletion and oxidative stress in a rat model of endotoxemia-induced ALI (4, 28). Additionally, this group recently characterized the effects of chronic ethanol pre-exposure on endotoxemia-induced ALI (35). Therefore, experimental endotoxemia with chronic ethanol pre-exposure is a useful experimental model to investigate mechanisms by which alcohol increases risk and susceptibility to ARDS. Although this experimental mouse model has been characterized, mechanisms by which ethanol pre-exposure exacerbates endotoxemia-induced ALI remain poorly understood. It is proposed that building on knowledge of established mechanisms of alcohol-induced injury in the liver- a direct target organ of alcohol toxicity- may aid in exploration of mechanisms of alcohol-enhanced ALI.

4. Parallel mechanisms of injury in the liver and lung

A major focus of the Arteel group's recent research has been to investigate liver-lung interactions in the setting of chronic alcohol abuse, and this group has accordingly sought to develop and characterize animal models to study both systems simultaneously. Indeed, the liver and lung share many

mechanisms of injury. First, resident macrophages, e.g., Kupffer cells in the liver and alveolar macrophages in the lung, play key roles in mediating the inflammatory response via the release of inflammatory cytokines. Alcohol-induced damage to both organs also appears to involve oxidative stress (4, 36). Furthermore, alcohol appears to enhance damage caused by a second “hit,” or inflammatory stimulus in both organs (37-40). For example, damage caused by intraperitoneal injection of LPS is enhanced by alcohol consumption in both the liver and the lung, as discussed briefly in Section 3 of this Chapter (28, 41, 42). Finally, an altered extracellular matrix (ECM) profile appears to be a key feature of pre-fibrotic inflammatory injury in both tissues. This pre-fibrotic, inflammatory remodeling of the ECM which does not alter the overall structure of the organ is a concept known as “transitional ECM remodeling” (43, 44). This group has demonstrated that the hepatic ECM responds dynamically to alcohol exposure, sensitizing the liver to LPS-induced inflammatory damage (38). Similarly, data from the laboratory of Jesse Roman, has demonstrated that chronic alcohol exposure alters the expression and degradation of the ECM, favoring fibronectin deposition (40). Although alcohol-induced liver and lung injury are by no means synonymous, they share many mechanisms of alcohol-induced organ injury, and understanding of one system can be used to leverage novel questions in the other.

5. Coagulation and fibrin ECM remodeling in alcohol-induced organ injury

A predominant theme of this dissertation is to explore a potential role of established mechanisms of alcohol-induced liver injury in the setting of alcohol-enhanced ALI. As discussed in Section 4 of the current Chapter, one potential parallel mechanism of liver and lung injury in response to alcohol is the development of a transitional ECM. To re-iterate, the “transitional ECM” can be defined as alterations in the amount or composition of the ECM that occur in the inflammatory, pre-fibrotic stages of disease that do not alter the overall structure or function of the organ (44). Fibrin(ogen) is one such transitional ECM protein in particular that is known to be affected by exposure to alcohol. Plasminogen activator inhibitor-1, or PAI-1, is a key regulator of fibrin degradation (i.e., fibrinolysis). PAI-1 is an acute phase protein normally expressed by adipocytes and endothelial cells, but is also expressed by other cells, including macrophages, during times of inflammation and/or stress (45). Classically, PAI-1 is an inhibitor of tissue-type plasminogen activator and urokinase-type plasminogen activator (tPA and uPA, respectively), thereby preventing the conversion of plasminogen to plasmin. Plasmin is a protease which degrades insoluble fibrin to fibrin degradation products. Thus, PAI-1 induction negatively regulates fibrinolysis.

The Arteel group has identified the critical role of PAI-1 in the progression of alcohol-induced liver injury. Specifically, knocking out PAI-1 produced potent anti-inflammatory effects in a chronic enteral alcohol model (46). Additionally, data from the Arteel group has also revealed a key role for PAI-1 in acute

alcohol-induced liver injury (38). In this setting, acute ethanol pre-exposure enhanced LPS-induced fibrin deposition in the hepatic sinusoidal space, and inhibiting fibrin deposition protected against enhanced liver injury and inflammation. Similar effects have also been demonstrated in other models of hepatic inflammation, including LPS-induced liver injury enhanced by partial hepatectomy (47). PAI-1 is also known to contribute to the development of ALI and ARDS (48, 49), and elevated plasma PAI-1 levels in patients with these conditions is associated with increased mortality (50-52). PAI-1 induction has also enhances susceptibility to LPS-induced ALI (53). PAI-1 induction also exacerbated fibrosis in a model of experimental, bleomycin-induced pulmonary fibrosis (54). Although the role of PAI-1 has been studied in many models of lung injury, including cancer (55), fibrosis (56), and ALI (57), the role of PAI-1 in alcohol-enhanced ALI remains largely unknown.

PAI-1 also indirectly regulates the expression of many other ECM proteins, including laminin, proteoglycan, and type IV collagen via inhibition of matrix metalloproteinases (MMPs) (58-61) (62). PAI-1 induction has also been shown to enhance fibronectin ECM deposition (63). Many cell types, including alveolar macrophages, bind to select ECM proteins under basal conditions, and cell surface receptors known as integrins mediate crosstalk between the ECM and the cell (64). For example, fibrin interacts with RGD-binding integrins, including integrin $\alpha_v\beta_3$. The Arteel group has demonstrated that blocking fibrin-integrin interactions protects against alcohol-induced liver injury with no effect on

fibrin accumulation (65), suggesting that this integrin signaling is critical for the progression of liver injury.

Altered integrin signaling may also contribute to injury in the lung. Alveolar macrophages, for example bind with high affinity to fibronectin via the integrins $\alpha_5\beta_1$. Chronic alcohol exposure has been shown to cause deposition of a fibronectin-enriched pulmonary ECM. Data from the Roman group has demonstrated that monocytes cultured on ECM from ethanol-exposed alveolar type II cells have an enhanced pro-inflammatory phenotype, and that blocking integrin $\alpha_5\beta_1$ -mediated monocyte-fibronectin interactions attenuates this pro-inflammatory phenotype (62). Ligation of integrin $\alpha_v\beta_3$, an integrin receptor for fibrinogen, vitronectin, and other ECM proteins that may be altered by alcohol has also been shown to be pro-inflammatory (66). PAI-1 may alter integrin signaling, both by promoting accumulation of integrin ligands, such as fibrin(ogen), as well as more directly inhibiting ECM-integrin interactions. For example, PAI-1 induction can inhibit vitronectin-integrin interactions, impairing tissue repair (67). Taken together, these data suggest that changing the ECM substratum can alter integrin signaling, and subsequently may promote inflammation and injury in a tissue. This dissertation will explore the hypothesis that PAI-1-mediated transitional remodeling of the pulmonary ECM, specifically accumulation of fibrin, may contribute to alcohol-enhanced acute lung injury, potentially via altered ECM-integrin signaling.

6. Murine models of alcohol exposure

At this point, it becomes necessary to explore the strengths and limitations of experimental models that may be used to investigate the questions presented in this dissertation. Murine models of alcohol exposure are one of the most valuable tools available to study the toxic effects of alcohol exposure, due to their relatively low maintenance cost and the wide prevalence of genetically modified strains available. Many potential routes of alcohol exposure are available in rodent models, including intravenous or intraperitoneal administration, as well as inhalation. While these models may be useful for investigating other pathologies associated with alcohol abuse, such as addiction, they produce little to no relevant alcohol-associated pathology, such as liver injury, in mice (68). Moreover, alcohol exposure in humans is overwhelmingly via an oral route. Therefore, oral administration of alcohol to mice is most commonly utilized to investigate alcohol-induced organ toxicity.

Murine models of oral alcohol exposure address different patterns of alcohol abuse, including acute (binge) drinking, as well as chronic abuse. Acute alcohol exposure models typically involve administration of ethanol (4-6 g/kg) via oral gavage over the course of 1-3 days. Pathologic changes (e.g., hepatic lipid accumulation and inflammatory cell recruitment) in acute ethanol models are typically minimal, yet these models can be useful for detecting biochemical (e.g., alterations in lipid/glucose metabolism) caused by alcohol (69). Furthermore, acute alcohol exposure, at doses that are not overtly toxic themselves, enhances the toxicity of other compounds, such as xenobiotics or bacterial endotoxins

(e.g., LPS). For example, in the liver, acute alcohol administration exacerbates damage caused by LPS (41). In the lung, acute alcohol exposure has been shown to exacerbate pulmonary congestion and inflammation in a model of burn injury (70).

Chronic administration of ethanol to mice is typically achieved by *ad libitum* feeding, either by ethanol in drinking water or a “forced choice” model of alcohol exposure, in which alcohol is incorporated into a liquid diet that serves as the only source of calories. In the lung, chronic administration of ethanol in drinking water (20% vol/vol for two weeks) depletes pulmonary glutathione levels and causes dysfunction of alveolar macrophages in mice (71). However, there are several concerns with utilization of this “ethanol in drinking water” mouse model, including low blood alcohol content (BAC), dehydration, and lack of an appropriate nutritional control (72). Furthermore, this model lacks significant manifestation of pathology in target organs, such as the liver. Indeed, Best et al. observed in 1949 that there was “no more evidence of a specific toxic effect of pure ethyl alcohol upon liver cells than there is for one due to sugar” (73). Based on these observations, it was proposed at the time that ALD was due to nutritional deficiencies rather than to alcohol itself.

To address these concerns, Lieber and DeCarli developed the first “forced-choice” model of chronic alcohol exposure, in which rats were administered a nutritionally complete ethanol-containing or iso-caloric maltose dextran-containing liquid diet for six weeks, with alcohol added in increasing concentrations up to 36% total calories (74). This feeding model at least partially

overcomes the rodent's natural aversion of alcohol, and thereby achieves higher BACs (typically around 0.10 g/dL) (75). Use of this model solidified the hypothesis that alcohol is indeed a direct hepatotoxin. In rats and mice, the Lieber-DeCarli liquid diet produces hepatic steatosis, mild elevations in plasma transaminases, and some necroinflammatory changes in the liver. In the lung, chronic alcohol exposure via ethanol containing liquid diet depletes glutathione levels (76), causes remodeling of the ECM (62), causes mitochondrial dysfunction (77), and sensitizes the lung to endotoxemia-induced ALI (35). Although these mechanisms of injury are important for understanding how chronic alcohol abuse sensitizes the lung to a second injury, this model does not produce overt histologic injury or inflammation in the lung.

A more robust model of chronic alcohol exposure involves enteral administration of ethanol-containing liquid diet via surgical implantation of an intragastric pump. In rodents, this feeding model produces micro- and macro-vesicular steatosis, apoptosis, inflammatory cell infiltration, and focal necrosis, making this model one of the most relevant for producing pathologies associated with ALD in humans (78). This model also produces ethanol-mediated toxicity in other organs, such as the pancreas (79). The effects of enteral alcohol exposure on other target organs, including the lung, is unknown. However, the technical skill required for the surgical implantation of the pump used in this model limits its widespread implementation.

Recently, the laboratory of Dr. Bin Gao developed the chronic + binge mouse model of alcohol exposure (80). In this model, mice are acclimatized to

control (0% ethanol) Lieber-DeCarli liquid diet for 5 days, followed by 10 days of 5% (v/v) ethanol-containing or iso-caloric control liquid diet. Then, animals receive a single binge (5 g/kg) of ethanol. This model more accurately reproduces drinking patterns of alcoholic patients who typically have a history of chronic drinking coupled with episodes of binge drinking. In animals, this alcohol exposure model produces high BACs (approximately 0.4 g/dL), elevations of plasma transaminases, and steatohepatitis which is predominately neutrophilic in nature, similar to what is observed in patients with alcoholic hepatitis. Furthermore, this model requires only moderate technical skill, and is relatively low-cost. Overall, this mouse model reproduces pathologies seen in human alcoholic patients and is relatively simple to perform, making it a useful experimental model for addressing questions relating to alcohol toxicity in target organs. However, the effects chronic + binge alcohol exposure on target organs other than the liver, such as the lung, have not been characterized.

7. Organ-organ crosstalk during alcohol exposure

As discussed throughout the current Chapter, alcohol abuse affects several organs and cell types in the body. For example, end-stage, decompensated liver disease is widely recognized as a systemic disorder. In fact, in patients with end-stage liver disease, cause of death is usually due to multiple organ failure, rather than liver disease itself (81). In one recent study of patients with alcoholic hepatitis (AH), 36% of patients developed multiple organ failure (82). In addition to the liver, kidney failure was found to be the most

common, followed by circulatory failure, coagulopathy, respiratory failure, and neurological failure. Ultimately, 90-day mortality in patients with multiple organ failure was found to be significantly higher than those without. Furthermore, patients with AH are also susceptible to the development of systemic inflammatory response syndrome (SIRS). This condition has also been associated with increased mortality. Indeed, multiple organ failure and widespread, systemic inflammation, is a hallmark of the end stage of alcoholic liver disease.

In addition to the end stage of disease, communication between the liver and other organs may be involved in the progression of disease in earlier, (i.e., inflammatory) stages. A primary goal of this dissertation is to investigate organ-organ interactions in the setting of chronic alcohol abuse, specifically, interactions between the liver and lung. The idea of the liver-lung axis in the setting of chronic alcohol exposure is based on clinical data demonstrating that patients with a diagnosed alcohol use disorder have increased incidence of and mortality from acute respiratory distress syndrome (ARDS), and in ARDS patients with hepatic failure, mortality increases to almost 100% (83). More recent data have indicated that even early liver dysfunction in ARDS is associated with worse prognosis in these patients (84). Additionally, experimental data suggest that communication with the liver may be required for lung injury in ALI models. For example, pulmonary injury induced by LPS can be altered by mediators released from the liver (e.g., $\text{TNF}\alpha$). Indeed, in an elegant

study by Siore and colleagues, LPS-induced lung damage required perfusion through the liver (85).

In a more recent study from the Arteel group, mice were exposed to chronic ethanol on the Lieber DeCarli liquid diet for six weeks, followed by intraperitoneal injection of LPS to produce endotoxemia-induced ALI (35). The differential effects on cytokine expression in systemic circulation and locally in the lung (i.e., in the BALF) were examined. Animals pre-exposed to ethanol diet had significantly elevated levels of plasma TNF α after LPS injection compared to animals fed a control diet. TNF α levels in the BALF, however, were unaffected, yet ethanol pre-exposed animals had elevated levels of the TNF α -responsive chemokines, MIP-2 and KC. This elevated chemokine expression also correlated with increased pulmonary neutrophil recruitment. Interestingly, blocking systemic TNF α using a TNF α -inhibiting antibody, etanercept, significantly attenuated the alcohol-enhanced pulmonary chemokine expression, and ultimately, alcohol-enhanced lung injury and inflammation after LPS. While the liver is not the sole source of systemic TNF α in this experimental setting, other studies have demonstrated that ablation of Kupffer cells shows that these cells are in fact a predominate source of plasma TNF α in experimental endotoxemia (86). These data, however, merely suggest a liver-lung axis in this disease state. Targeted animal models to specifically investigate the role of liver-derived mediators are not yet available. Therefore, a major goal of this dissertation is to develop a more specific system to directly examine the role of hepatic-derived cytokines in alcohol-enhanced pulmonary injury.

8. Statement of goals

As discussed throughout this chapter (Section 4), the liver and the lung share many parallel mechanisms of injury in response to alcohol exposure, such as transitional remodeling of the ECM. Furthermore, as discussed in Section 7, the liver and lung may communicate during the development of disease.

Therefore, the goal of this dissertation is to expand on established mechanisms of alcohol-induced liver injury to ask innovative questions about mechanisms of alcohol-enhanced acute lung injury, as well as to develop new tools that may be used to gain novel insight into the liver-lung axis of alcohol-induced injury. These goals will be discussed in detail in the following Section.

B. Aims and proposals

1. Investigating the role of PAI-1 in alcohol-enhanced acute lung injury: parallel mechanisms of liver and lung injury

This dissertation proposes that exploring parallel mechanisms of liver and lung injury in response to chronic alcohol exposure will enhance the field's understanding of alcohol-induced organ injury as a whole, as well as provide new targets for therapy. Recent work in the Arteel laboratory indicates that activation of plasminogen activator inhibitor-1 (PAI-1) and subsequent fibrin ECM remodeling drives inflammatory liver damage in alcohol-induced liver injury. Previous studies have established that PAI-1-mediated ECM remodeling contributes to inflammatory damage in several organs, including lung. However,

the role of PAI-1 in alcohol-enhanced ALI remains unknown. It is hypothesized that PAI-1 induction and PAI-1-mediated fibrin accumulation in the lung is critical for the progression of alcohol-related lung inflammation. The goals of the first Aim of this dissertation are therefore 1) to establish if PAI-1, as well as downstream targets of PAI-1 (e.g., fibrin) are up-regulated in a two-hit mouse model of chronic alcohol exposure and experimental endotoxemia, 2) to determine the role of PAI-1 in injury and inflammation in this model using PAI-1 knockout mice, and 3) to establish a potential mechanism by which PAI-1 and fibrin are involved in alcohol-enhanced ALI.

2. Characterizing lung injury in a clinically relevant mouse model of “chronic + binge” alcohol exposure

A major focus of research in the Arteel group is to understand how alcohol abuse affects the organism as a whole, including multiple target organs simultaneously. One common limitation of the models typically employed to study the effects of alcohol on the lung is the absence of relevant pathology in other target organs, such as the liver. Indeed, the ethanol in drinking water model, which is commonly used to study alcohol-induced lung toxicity, has been demonstrated to cause little to no liver pathology. Interestingly, only a small minority of patients with alcohol use disorders have histologically normal livers (87). Established in 2013, the “chronic + binge” (i.e. “NIAAA model” or “acute-on-chronic model”) is quickly becoming recognized as a more accurate representation of human alcohol abuse, particularly risky drinking behavior. This

model has been used extensively to study the development and progression of alcoholic (steato)hepatitis. However, the effects of acute-on-chronic alcohol exposure on the lung are unknown, although risky drinking behavior is clearly associated with ALI. Therefore, it is proposed that chronic + binge alcohol exposure may unmask alcohol-induced pathologies in the lung that are not seen with other, more moderate patterns of alcohol exposure. The goals of the second Aim of this dissertation are therefore 1) to characterize lung injury and/or inflammation in this clinically relevant model of alcoholic exposure, 2) to determine if acute-on-chronic alcohol exposure causes functional effects in the lung, and 3) to determine if alcohol exposure alone, absent any secondary injury is sufficient to cause pulmonary inflammation.

3. Establishing a transgenic animal system to investigate communication between the liver and lung during alcohol exposure

Recent work from this group has demonstrated that the liver and lung not only share parallel mechanisms of alcohol-induced injury, but that hepatic-derived cytokines (TNF α) may, at least in part, contribute to lung injury. This group showed that EtOH-fed mice that were co-treated with etanercept, a TNF α inhibitor, showed significantly attenuated lung injury after LPS injection. However, these data are only suggestive of a liver-lung axis in alcohol-induced organ toxicity. The third Aim of this dissertation will build on these findings by attempting to develop an inducible transgenic animal in which genes of interest, (e.g., TNF α converting enzyme (TACE)) may be selectively deleted from hepatic

macrophages, thereby providing a mechanism to definitively test the hypothesis that hepatic-derived inflammatory mediators play a significant role in alcohol-enhanced ALI. Furthermore, this transgenic system will have limitless applications in a wide variety of disease models. The goals of this Aim are therefore 1) to identify a drug-delivery method which selectively targets the liver versus the lung, and 2) to develop a transgenic animal to demonstrate the feasibility of selectively inducing a transgene in the liver versus the lung.

Overall aim of this dissertation

The overall aim of this dissertation is to expand on the unifying hypothesis that not only do the liver and lung share parallel mechanisms of injury in response to alcohol exposure, but that injury in these two systems may be interdependent. For example, dysregulation of fibrinolysis has been demonstrated to be critical in the development of alcohol-induced liver injury, as well as in various models of ALI. However, the effect of PAI-1 and subsequent fibrin accumulation on alcohol-enhanced ALI is not known. This question will be addressed in Aim 1. Additionally, recent data from this group, as well as others, have suggested that communication between the liver and lung, possibly via hepatic-derived inflammatory cytokines like TNF α , may contribute to alcohol-enhanced injury and inflammation in the lung. The pulmonary effects of a well-established, relevant model of alcoholic liver disease, would therefore be of interest to establish. This question will be the primary goal of Aim 2 of this dissertation. Finally, although recent data with the TNF α inhibitor etanercept

suggests that hepatic-derived TNF α contributes to alcohol-enhanced ALI, an empirical system by which to address this mechanistic link remains unestablished. Aim 3 will seek to establish an inducible transgenic animal model to interrogate this hypothesis. Taken together, this dissertation will build novel insight in the liver-lung axis of disease.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. Animals and Treatments

Mice were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the University of Louisville's Institutional Animal Care and Use Committee.

1. Animal sacrifice, tissue collection and storage

At time of sacrifice, animals were anesthetized with ketamine/xylazine (100/15 mg/kg, i.p.). Blood was collected from the vena cava just prior to sacrifice by exsanguination and citrated plasma was stored at -80 °C for further analysis. Prior to lavage of the lungs, 10 mL of sterile PBS was perfused through the heart to flush the pulmonary vasculature of erythrocytes. Bronchoalveolar lavage (BAL) was performed by flushing the lung two times with 400 µL sterile PBS. Cells in the BALF were separated by centrifugation and removed from remaining BALF and fixed on slides for further analysis (88). Portions of tissue (liver, lung, kidney, and spleen) were snap-frozen in liquid nitrogen for later analysis or fixed in 10% neutral buffered formalin for subsequent sectioning and

mounting on microscope slides. Total RNA was immediately extracted from fresh lung tissue using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) and chloroform:phenol separation (see section D for additional details).

2. Chronic model of alcohol exposure

Eight week old male C57BL6/J, *Pai-1* knockout (B6.129S2-Serpine1^{tm1Mlg/J}: PAI-1^{-/-}), and integrin β_3 knockout (B6.129S2-Itgb3^{tm1Hyn/J}SemJ: $\beta_3^{-/-}$) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and exposed to either ethanol-containing Lieber-DeCarli diet (Dyets, Inc., Bethlehem, PA) or iso-caloric control diet. During the exposure period, animals were housed in pairs in shoebox cages in a room held at 75 °F. Diet was provided in vacuum tubes and replaced between 4:00 and 5:00 PM daily. Both ethanol-fed animals and their pair-fed counterparts received control diet for the first two days of liquid diet feeding to allow acclimation to the liquid diet feeders. After 2 days of acclimation, ethanol concentrations were increased incrementally over the course of three weeks before reaching the highest ethanol concentration, 6% (vol/vol) for the final three weeks of exposure. Ethanol concentrations in the ethanol-containing diets were as follows: 0% for two days of acclimation, 1% for two days, 2% for two days, 4% for one week, 5% for one week, and 6% for three weeks. Ethanol-containing diet was provided *ad libitum* for the entire course of the study. Because of the relatively high caloric content of ethanol, pair-fed control animals received an iso-caloric control diet; the calories in the iso-caloric diet were matched by adding a calorie-equivalent of maltose-dextran. To

account for the reduced food consumption of ethanol-fed mice, pair-fed mice were given the volume of diet consumed by their ethanol-fed counterparts the night before. At the conclusion of the feeding period, the two diet groups were further separated into additional groups that received either LPS (*E. coli*; 10 mg/kg i.p Sigma-Aldrich, St. Louis, MO) or vehicle (saline). Animals were euthanized 4 or 24 h after LPS (or vehicle) injection, and samples were collected as described in section A1.

3. Chronic + binge model of alcohol exposure

Ten-week-old C57Bl6/J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were treated as described by Bertola et al (80). During the exposure period, animals were housed in pairs in shoebox cages in a room held at 75 °F. Diet was provided in vacuum tubes and replaced between 4:00 and 5:00 PM daily. Animals were acclimatized to control (0% EtOH) Lieber-DeCarli liquid diet (Dyets, Inc., Bethlehem, PA) for 5 days. Mice were then split into two groups to ethanol-containing (5% v/v) or iso-caloric control liquid diet for 10 days. To account for the reduced food consumption of ethanol-fed mice, pair-fed mice were given the volume of diet consumed by their ethanol-fed counterparts the night before. On day 11, mice received ethanol (5 g/kg) or iso-caloric maltose dextran binge via oral gavage. Mice were sacrificed 9 or 24 hours post-binge. Tissues were collected as described in section A1.

4. Generation of transgenic mice

Female mice homozygous for tamoxifen-inducible Cre recombinase (B6;129-Gt(ROSA)26Sor^{tm1(cre/ERT)}Nat/J or simply, R26CreER) and male mice homozygous for a two-color fluorescent Cre reporter allele (B6.129(Cg)-Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)}Luo/J, or simply ROSA^{mT/mG}) were purchased from the Jackson Laboratory (Bar Harbor, ME). The two-color fluorescent reporter allele used in these experiments, also known as ROSA^{mT/mG} expresses cell membrane-targeted red fluorescence (tdTomato) ubiquitously prior to exposure to Cre recombinase. Upon Cre-mediated recombination, the tdTomato cassette, which is flanked by lox-p sites, is excised, allowing for expression of membrane-targeted enhanced green fluorescent protein (EGFP) located downstream.

Female homozygous R26CreER mice were crossed with male ROSA^{mT/mG} mice at 8-10 weeks of age to produce mice heterozygous for these two transgenes (R26CreER⁺/mTmG⁺, F1 generation). All pups were weaned at post-natal day 21. Animals used in future experiments were between 6-12 weeks of age.

5. Intraperitoneal administration of tamoxifen

Tamoxifen (Sigma-Aldrich, St. Louis, MO) was injected in R26CreER⁺/mTmG⁺ mice according to a protocol described by The Jackson Laboratory (Bar Harbor, ME). Briefly, tamoxifen was dissolved in corn oil (20 mg/mL) by shaking overnight in a light-protected vessel at 37 °C. Tamoxifen was

then injected at a final concentration of 75 mg/kg*bw once every 24 hours for a total of 5 consecutive days. Mice were sacrificed 7 days after the final injection as described in Section A.1 and B.6.

6. Intravenous administration of tamoxifen-loaded PLGA nanoparticles

Poly(lactic-co-glycolic acid) (PLGA) nanoparticles were prepared in 200 μ L sterile saline at a final concentration of 1.5 mg nanoparticles per 25 g of body weight. Just prior to injection, nanoparticles were sonicated in a water bath sonicator for 1 minute to break up any nanoparticle clusters. Mice were restrained, and nanoparticles were injected with a 28 gauge insulin syringe in the tail vein after warming the tail with a heat lamp. Mice were sacrificed 7 days after the final injection as described in Section A.1 and B.6.

7. Intrasplenic administration of tamoxifen-loaded PLGA nanoparticles

PLGA nanoparticles were administered via intrasplenic injection in mice as described elsewhere (89) under aseptic conditions. Briefly, mice were anesthetized with inhaled isofluorane and abdominal hair was removed. After laparotomy, the spleen was visualized and PLGA nanoparticles were injected with a 28 gauge insulin syringe at a dose of 0. 0.375, 0.75, or 1.5 mg per 25 g of body weight in 200 μ L sterile saline. Just prior to injection, nanoparticles were sonicated in a water bath sonicator for 1 minute to break up any nanoparticle clusters. Antibiotics (penicillin [10,000 Units/mL] streptomycin [10,000 μ g/mL]) (Life Technologies, Carlsbad CA) were administered intraperitoneally (50 μ L) and

the incision was closed using 3-0 silk sutures. Animals were singly housed after the procedure and sacrificed as described in Section A.1 7 days later. Tissues were collected as described in Section A.1 and B.6.

B. Histology

1. General morphology

Formalin fixed, paraffin embedded liver and lung tissues were cut at 5 μ m and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Thermo Fisher Scientific, Waltham, MA) and rehydrated through graded ethanol. Sections were then stained with hematoxylin and eosin (H&E). After staining, samples were dehydrated through graded alcohol, washed in Citrisolv and then mounted with Permount (Thermo Fisher, Waltham, MA).

2. Scoring of alveolar septal thickening

Twenty blinded photomicrographs per sample of hematoxylin and eosin-stained paraffin embedded lung tissues were taken in successive fields at 400x magnification and saved. Four randomly selected alveolar septa per photograph were measured using the straight line tool on ImageJ software (90). Resulting measurements are given as length of the straight line pixels and represented as fold of control. As per guidelines established by the American Thoracic Society, areas of the tissue that were underinflated during histological preparation were avoided, as this may cause the septa to appear artificially thickened (29). Additionally, septa directly adjacent to a blood vessel or airway were avoided, as

these septa are normally thickened by collagen present in the peribronchovascular bundle (29).

3. Neutrophil accumulation

Neutrophil accumulation in lung tissue was measured using chloracetate esterase (CAE) staining. Briefly, formalin fixed, paraffin embedded lung tissue was cut at 5 μ m and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Thermo Fisher Scientific, Waltham, MA) and rehydrated through graded ethanol. Tissue specimens were incubated in a solution of naphthol AS-D chloroacetate (1 mg/ml) in N,N-dimethylformamide, with 4% sodium nitrite and 4% new fuchsin (Sigma-Aldrich, St. Louis, MO). The naphthol AS-D chloroacetate is enzymatically hydrolyzed by chloroacetate esterase in neutrophils, liberating the naphthol compound. Naphthol combines with a freshly-formed diazonium salt, leaving bright pink color deposits at the site of enzymatic activity (University of Calgary Airway Inflammation Research Group).

4. Immunohistochemistry

Immunohistochemistry for CD41 was performed by the Michigan State University Investigative Histopathology Laboratory. Previously sectioned slides were subsequently de-paraffinized in xylene and hydrated through descending grades of ethyl alcohol to distilled water. Slides were placed in Tris Buffered Saline pH 7.4 (Scytek Labs, Logan, UT) for 5 minutes for pH adjustment. Following TBS, slides underwent heat retrieval utilizing Scytek Citrate Plus

Retrieval pH 6.0 in a vegetable steamer for 30 minutes at 100°C, allowed to cool on the counter at room temperature for 10 minutes and rinsed in several changes of distilled water. Endogenous Peroxidase was blocked utilizing 3% Hydrogen Peroxide / Methanol bath for 30 minutes followed by running tap and distilled water rinses. Following pre-treatment standard micro-polymer complex staining steps were performed at room temperature on the IntelliPath™ Flex Autostainer. All staining steps are followed by rinses in TBS Autowash buffer (Biocare Medical, Concord, CA). After blocking for non-specific protein with Background Punisher (Biocare) for 5 minutes; sections were incubated with Rabbit Polyclonal anti - CD41/integrin α_{IIb} (Abcam ab83961, Cambridge, MA) @ 1:100 in normal antibody diluent (NAD-Scytek) incubated for 2 hours. Mach 3 Probe and Mach 3 Polymer™ anti rabbit reagents (Biocare) were applied for 10 minutes each followed by reaction development utilizing Romulin AEC™ (Biocare) for 5 minutes and counterstain with (Biocare) Cat Hematoxylin for 5 minutes. Slides were visualized on a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan), and DAB staining was quantified in ten high-magnification (400x) fields using Metamorph software (Molecular Devices, Sunnyvale, CA).

Green fluorescent protein (GFP) was detected immunohistochemically in liver and lung sections. Previously sectioned formalin-fixed, paraffin-embedded liver and lung sections were deparaffinized and heat-mediated antigen retrieval was performed in 0.01 M sodium citrate (pH 6.0) in a vegetable steamer. Following antigen retrieval, endogenous peroxidases were quenched in 3% hydrogen peroxide. Blocking for endogenous biotin was performed using a

commercially available kit (Agilent Technologies, Santa Clara, CA). Prior to applying primary antibody, sections were blocked in 10% goat serum in PBS. Sections were incubated in 1:200 rabbit anti-GFP (Cell Signaling Technologies 2956S, Danvers, MA) overnight at 4 °C. The Vectastain Elite ABC kit was used for detection (Vector Laboratories, Inc., Burlingame, CA). Briefly, tissues were incubated in a biotinylated anti-rabbit IgG secondary antibody for 30 minutes at room temperature. Tissues were washed and incubated in a solution containing avidin-bound horseradish peroxidase (HRP) for 30 minutes. The HRP substrate 3, 3'-diaminobenzidine (DAB) (Agilent Technologies, Santa Clara, CA) was added to sections until positive (brown) staining was macroscopically visible. Slides were counterstained with hematoxylin for 1 minute, washed, dehydrated through graded ethanol and then mounted with Permount (Thermo Fisher, Waltham, MA). Each slide contained a negative tissue section that did not receive primary antibody. Slides were visualized using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) with Metamorph software (Molecular Devices, Sunnyvale, CA).

5. Immunofluorescence

Immunofluorescent detection of fibrin accumulation has been described previously (38), and was conducted with minor modifications. Previously sectioned lung tissues (5 μ M) were deparaffinized with Citrisolv (Thermo Fisher, Waltham, MA) and rehydrated through graded ethanol. To quench autofluorescence of paraffin-embedded tissue, tissue sections were incubated in

70% EtOH containing 0.25% NH₄OH for 1 hour at room temperature during deparaffinization. Proteolytic digestion was performed by incubating deparaffinized tissue sections using 0.03% Pronase E (Sigma-Aldrich, St. Louis, MO) for 10 minutes at 37° C, then sections were incubated in sodium borohydride (10 mg/mL) for 40 minutes at room temperature for additional autofluorescence quenching. To minimize non-specific binding of the antibody, sections were incubated in 10% goat serum in PBS for 30 minutes at RT. Sections were then incubated with rabbit polyclonal anti-fibrinogen (Agilent Technologies A0080, Santa Clara, CA) in blocking buffer (1:1000) overnight at 4 °C. After washing in PBS, sections were incubated with AlexaFluor 488 goat anti-rabbit secondary antibody (1:500) (Life Technologies, Carlsbad, CA) in blocking buffer for 3 hours at RT. After washing, slides were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Labs, Burlingame CA). Slides were visualized a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) with Metamorph software (Molecular Devices, Sunnyvale, CA).

6. Visualization of red and green fluorescence in frozen tissue sections

For visualization of TdTomato red fluorescent protein (RFP) and enhanced green fluorescent protein (EGFP) native fluorescence in tissues, mice were perfused through the heart with ice cold 4% paraformaldehyde (PFA) in 0.1 M PBS. Tissues were fixed in 4% PFA in 0.1 M PBS for 24 hours. After fixing, tissues were cryoprotected in 30% sucrose solution for 24 hours, then embedded

in Tissue-Plus Optimal Cutting Temperature (OCT) embedding medium (Thermo Fisher, Waltham MA) by freezing in liquid nitrogen. After embedding, samples were allowed to come to -20 °C and sectioned at 5 µM on a Leica cryostat. Sections were air-dried for 1 hour at RT, washed 3 times in PBS, and mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Labs, Burlingame CA). Slides were visualized using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) with Metamorph software.

7. Differential staining of BAL cells

Cells in BAL fluid were counted using a hemocytometer, and cells were spun onto glass slides using a Cytospin centrifuge (Thermo Fisher Scientific, Waltham, MA). Cells were stained with the Shandon Kwik-Diff (Thermo Fisher Scientific, Waltham, MA) differential staining kit according to manufacturer's instructions. Slides were visualized on a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) with Metamorph software (Molecular Devices, Sunnyvale, CA), and total number of cells in 2 low-magnification (100x) fields were counted using ImageJ software.

C. Clinical chemistry

1. Plasma transaminase activity

Plasma activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (Thermo Fisher Scientific, Waltham, MA).

2. Myeloperoxidase Activity

Myeloperoxidase activity was measured as previously described (91, 92). Frozen lung tissue samples were thawed and homogenized in 50 mM potassium phosphate buffer (pH 6.0), then centrifuged at 9000 x g for 15 minutes at 4°C. Supernatant (containing hemoglobin) was discarded, and the resulting pellet was resuspended in potassium phosphate buffer (pH 6.0) containing 50 mM hexadecyltrimethylammonium bromide (HTAB) and homogenized again. This suspension was sonicated, and snap frozen in liquid nitrogen and thawed for three freeze-thaw cycles. Samples were then centrifuged at 9000 x g for 10 minutes at 4°C. The resulting supernatant was then assayed spectrophotometrically for myeloperoxidase activity in 50 mM potassium phosphate (pH 6.) with 0.0005% hydrogen peroxide and 0.167 mg/mL o-dianisidine dihydrochloride (substrates of myeloperoxidase) at 450 nm. Results are reported as rate of reaction per mg of lung tissue.

3. BALF total protein measurement

BALF from treated animals was collected as described previously. Total protein in BALF was measured spectrophotometrically using a modified Lowry Assay (Bio-Rad DC Protein Assay, Bio-Rad Laboratories, Hercules CA) according to manufacturer's instructions.

4. ELISA

Plasma thrombin-antithrombin (TAT) complex levels were detected in citrated plasma as previously described (38, 93) using a commercially available ELISA kit (Dade Behring Inc., Deerfield, IL).

D. RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from lung tissue by a guanidinium thiocyanate-based method (RNA STAT-60, Tel-Test, Inc., Friendswood, TX). RNA concentrations were determined spectrophotometrically and 1µg of total RNA was reverse transcribed using a kit (Quantabio, Beverly, MA).

The pulmonary mRNA expression of select genes was detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), which is routine for the Arteel group (38). PCR primers and probes for *Tnf-α*, *Pai-1*, *Il-6*, and *Il-1β* were designed using Primer 3 (Whitehead Institute for Biomedical Research, Cambridge MA). Primers and probes for *Icam-1*, *Vcam-1*, *Pecam/Cd31*, *Cxcl2*, *Cxcl1*, *F4/80*, *Cxcl15*, *Ly6g*, *Cd68*, and *β-actin* were purchased from Life Technologies (Carlsbad, CA). All primers were designed to cross introns and ensure that only cDNA and not genomic DNA was amplified. PerfeCta qPCR Fast Mix (Quantabio, Beverly, MA) was added to the PCR mixture. This 2X mixture is optimized for TaqMan reactions and contains MgCl₂, dNTPs, and AccuFast Taq DNA Polymerase. Amplification reactions were carried out using the ABI StepOne Plus machine and software (Applied Biosystems, Foster City, CA) with initial holding stage (95°C for 30 seconds) and

50 cycles of a 2-step PCR (95°C for 30 seconds, 60°C for 20 seconds).

Fluorescent intensity of each sample was measured at each cycle to monitor amplification of the target gene. The comparative CT method was used to determine fold changes in mRNA expression compared to an endogenous reference gene (β -actin). This method determines the amount of target gene, normalized to an endogenous reference and relative to a calibrator ($2^{\Delta\Delta CT}$).

E. Pulmonary Mechanics Measurements

Pulmonary mechanics at baseline and in response to inhaled methacholine were measured in mice as described in (94). Measurements were performed by forced oscillation technique using a flexiVent system (SCIREQ, Montreal, Quebec, Canada). Mice were anesthetized with tribromoethanol (375 mg/kg i.p.). A cannula connected to a pressure transducer and ventilator was inserted into the trachea. Mice were placed on a warming plate and attached to EKG leads, and lungs were mechanically ventilated with air at a tidal volume of 6 ml/kg and a frequency of 150 breaths/min. To prevent endogenous breathing effort, mice were given pancronium bromide (0.8 mg/kg i.p.) every 20 min until the completion of airway reactivity measurements. During the experiment, mice were given additional tribromoethanol to maintain heart rate at or below the baseline level. Baseline pulmonary mechanics measurements were collected using 1) a single perturbation at 2.5 Hz to derive resistance and compliance based on the single compartment model, 2) a broadband perturbation from 1 to 20.5 Hz to derive frequency-dependent impedance and parameters based on the

constant phase model, and 3) quasi-static pressure-volume curves. Following baseline assessment, measurements were then repeated following administration of increasing doses of aerosolized methacholine generated from solutions of 0, 6.25, 12.5, 25, or 50 mg/mL methacholine. Methacholine was aerosolized for 10 seconds from an Aeronib nebulizer that delivered 0.15 mL/min and respiratory parameters were repeatedly collected for a total of 15 measurements of each parameter. For each methacholine dose the average of 15 measurements was collected.

F. Synthesis of PLGA nanoparticles

PLGA nanoparticles (NPs) encapsulating tamoxifen (Sigma-Aldrich, St. Louis, MO) were synthesized as previously described, using an oil-in-water (o/w) single emulsion technique (95-97). Briefly 100 mg tamoxifen was dissolved in 2 mL dichloromethane (DCM). In parallel, 100 mg poly(lactic-co-glycolic acid) (PLGA) carboxyl-terminated polymer (0.55-0.75 dL/g, LACTEL[®], DURECT Corp.) was dissolved in 1 mL of DCM. The PLGA and tamoxifen (200 μ L) solutions were combined to obtain a final theoretical loading of 200 μ g tamoxifen per mg of PLGA. The PLGA/tamoxifen/DCM solution was added dropwise to a 2 mL 5% polyvinyl alcohol (PVA) solution, then vortexed and sonicated. Residual DCM was evaporated by adding the NP solution to 50 mL of 0.3% PVA for 3 hrs while mixing. After solvent evaporation, the tamoxifen NPs were transferred to tubes and centrifuged at 20,444 x g at 4°C and washed 3 times in deionized water (diH₂O). NPs were frozen, lyophilized, and stored at -20°C until use.

G. Characterization of PLGA nanoparticles

Particle size and morphology were determined using scanning electron microscopy (SEM, JSM-820, JEOL). Dry NPs were mounted on carbon tape and sputter-coated with gold under vacuum. The average unhydrated NP diameters were determined from SEM images of at least 200 particles per batch using ImageJ image analysis software.

The amount of tamoxifen encapsulated within the NPs was quantified by dissolving 3-5 mg tamoxifen NPs and control blank NPs in dimethyl sulfoxide (DMSO) for 30 min. Subsequent dilutions were done to obtain samples within the linear range of a free tamoxifen standard in DMSO. The loading of tamoxifen, defined as the amount of tamoxifen incorporated per milligram of NP, was measured using UV absorption spectroscopy at 265 nm. Background measurements of blank NPs in DMSO were subtracted from tamoxifen NP sample readings. Encapsulation efficiency, or the percent Tamoxifen incorporated relative to the amount of tamoxifen initially loaded, was calculated as follows: $[(\text{Mass of tamoxifen incorporated per mg of NP}) / (\text{Mass of tamoxifen initially added to electrospinning solution per mg of NP})] \times 100$.

To determine the release profiles of the tamoxifen *in vitro*, triplicate NP samples of 3-5 mg were suspended in 1 mL PBS. NP samples were incubated at 37°C and constantly shaken. At each of the following time points: 1, 4, 8, 24, 48, 72, and 168 hrs, NPs were centrifuged to obtain a pellet (13,000 rpm), and the complete volume of PBS was removed and replaced with fresh PBS. Tamoxifen

released into PBS was quantified using absorption spectroscopy at 265 nm. All samples were analyzed in triplicate.

H. Statistical Analyses

Results are reported as means \pm standard error (SEM). Unless otherwise specified, one-way or two-way ANOVA with Bonferroni's post-hoc test (for parametric data) or was used for the determination of statistical significance among multiple treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study as the level of significance.

CHAPTER III²

PLASMINOGEN ACTIVATOR INHIBITOR 1 PLAYS A CRITICAL ROLE IN ALCOHOL-ENHANCED ACUTE LUNG INJURY IN MICE²

A. Introduction

Alcohol consumption is a common custom worldwide. In the United States alone, 87.6% of adults report consuming alcohol at some point in their lives (98). This widespread consumption of alcohol continues, despite the fact that the detrimental health effects of alcohol abuse are well-established. In fact, alcohol is known to contribute to the development of over 200 disease states (99), making alcohol the fifth leading risk factor for premature death and disability worldwide (100). The lung is recognized as a target of chronic alcohol abuse, and alcohol-related lung injury is estimated to account for tens of thousands of deaths in the United States each year (18). Although chronic alcohol consumption is not directly linked to the development of lung disease per se, it appears to sensitize the lung to damage from other causes. For example, acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), occurs 3.7 times more often in people meeting the diagnostic criteria for alcohol use disorders, and these patients have a worse prognosis (19-21).

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The exact mechanisms by which alcohol sensitizes the lung to ALI are incompletely understood. Although alcohol does not appear to directly cause overt histologic injury to the lung, experimental alcohol exposure induces expression of pro-inflammatory cytokines (22-24), activates tissue remodeling (26, 27), and increases oxidative stress in rodent lungs (28). These factors are hypothesized to contribute to the development of an “alcoholic lung” phenotype, which enhances the host’s susceptibility to serious lung diseases (18). Better understanding of this complex process could identify potential therapeutic targets to treat or prevent alcohol-related lung dysfunction, including enhanced susceptibility to ALI and ARDS.

Plasminogen activator inhibitor-1, or PAI-1, is a key regulator of the fibrinolytic system. PAI-1 is an acute phase protein normally expressed by adipocytes and endothelial cells, but is inducible in other cell types, including macrophages, during times of inflammation and/or stress (45). PAI-1 is readily induced by almost all stress signaling, including hypoxia, oxidative stress and inflammatory cytokines (101). PAI-1 is the primary physiological inhibitor of fibrinolysis. PAI-1 inhibits tissue-type plasminogen activator and urokinase-type plasminogen activator (tPA and uPA, respectively), enzymes that convert plasminogen to plasmin, which degrades fibrin. The Arteel group has demonstrated that PAI-1 is critical in experimental inflammatory liver injury caused by alcohol (38, 46). PAI-1 also plays a critical role in experimental ALI (54), and is suspected to contribute to the incidence and severity of ARDS in humans (50, 52). However, the contribution of PAI-1 in alcohol-enhanced ALI

has not been determined. The purpose of this study was to determine the role of PAI-1 in alcohol-enhanced inflammatory lung injury caused by LPS.

B. Experimental Procedures

1. Animals and treatments

Animals were administered ethanol-containing Lieber-Decarli liquid diet for 6 weeks and injected with LPS (See Scheme 1.1) as described in Chapter II, Section A.2. Upon sacrifice, tissues were collected as described in Chapter II, Section A.1.

2. Histology

Lung tissues were stained with hematoxylin and eosin for general morphology and scored as described in Chapter II, Sections B.1 and B.2. Neutrophil accumulation in tissues were visualized with chloracetate esterase staining as described in Chapter II, Sections B.3. CD41 and Fibrin were detected in paraffin-embedded lung sections as described in Chapter II, Sections B.4 and B.5.

3. Clinical chemistry

BALF total protein levels were measured as described in Chapter II, Section C.3. Plasma thrombin anti-thrombin (TAT) was detected using a commercially available ELISA kit, as described in Chapter II, Section C.4.

Myeloperoxidase activity in lung tissue was measured as described in Chapter II, Section C.2.

4. RNA Isolation and qRT-PCR

Details for RNA isolation and qRT-PCR are described in Chapter II, Section D.

5. Statistical analysis

Statistical analysis was performed as described in Chapter II.

C. Results

1. Chronic ethanol feeding enhances pulmonary PAI-1 expression and fibrin accumulation caused by LPS.

PAI-1 is proposed to play a role in models of ALI in the absence of alcohol (53, 54). Furthermore, PAI-1 is critically involved in alcohol-induced liver injury (47). Therefore, animals were treated with chronic ethanol-containing diet and LPS as described in Chapter II (Scheme 3.1.), and the effects of ethanol and LPS on pulmonary PAI-1 expression were determined (Figure 3.1A). LPS administration robustly increased the expression of *Pai-1* mRNA (~1000 fold, $p < 0.05$) in the lungs. Although, ethanol feeding alone did not affect *Pai-1* expression, it significantly enhanced the increase in *Pai-1* expression caused by LPS, with values ~2-fold higher than with LPS alone. PAI-1 protein levels in the BAL fluid (24 h after LPS) paralleled the pattern of mRNA expression (Figure 3.1A).

Scheme 3.1: Lieber-DeCarli model of chronic alcohol exposure and experimental endotoxemia

Mice received ethanol-containing or isocaloric maltose-dextrin containing Lieber-DeCarli diet (see Experimental Procedures) for six weeks and were injected with LPS (10 mg/kg i.p.) or vehicle (saline) 4 or 24 hours prior to sacrifice.

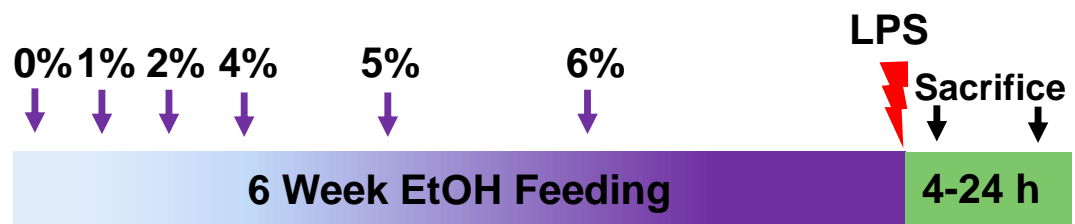
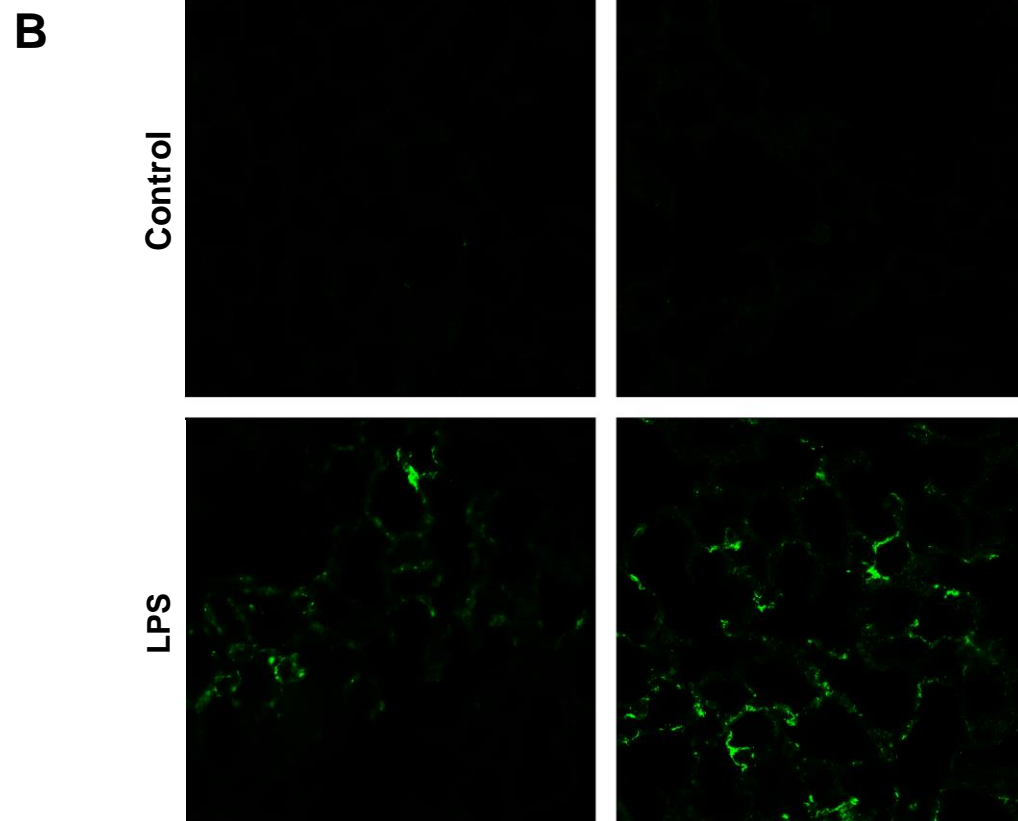
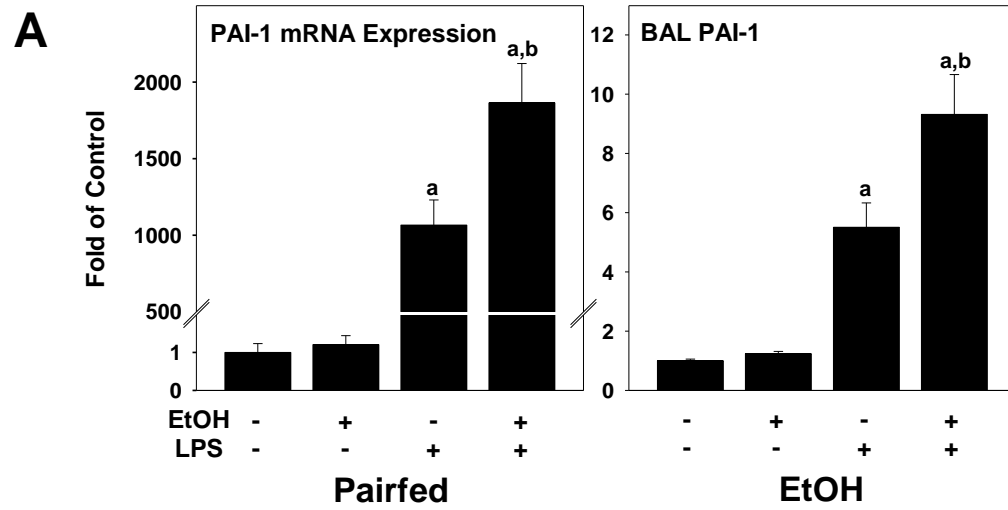


Figure 3.1. Effect of ethanol on LPS-induced pulmonary PAI-1 expression and pulmonary fibrin accumulation.

Pulmonary *Pai-1* mRNA expression measured by qRT-PCR and PAI-1 protein levels in BAL fluid. (B) Representative photomicrographs (400×) of pulmonary fibrin deposition detected immunofluorescently (green) in paraffin-embedded tissues 24 h after LPS injection. Results are reported as means ± standard error mean (SEM; n= 4-6), ^a, $p < 0.05$ compared to pair-fed control, ^b, $p < 0.05$ compared to LPS alone, ^c, $p < 0.05$ compared to WT animals. Appears in Poole LG et al. *Am J Respir Cell Mol Biol*. 2017 Apr 26. DOI: 10.1165/rcmb.2016-0184OC, reprinted with permission.



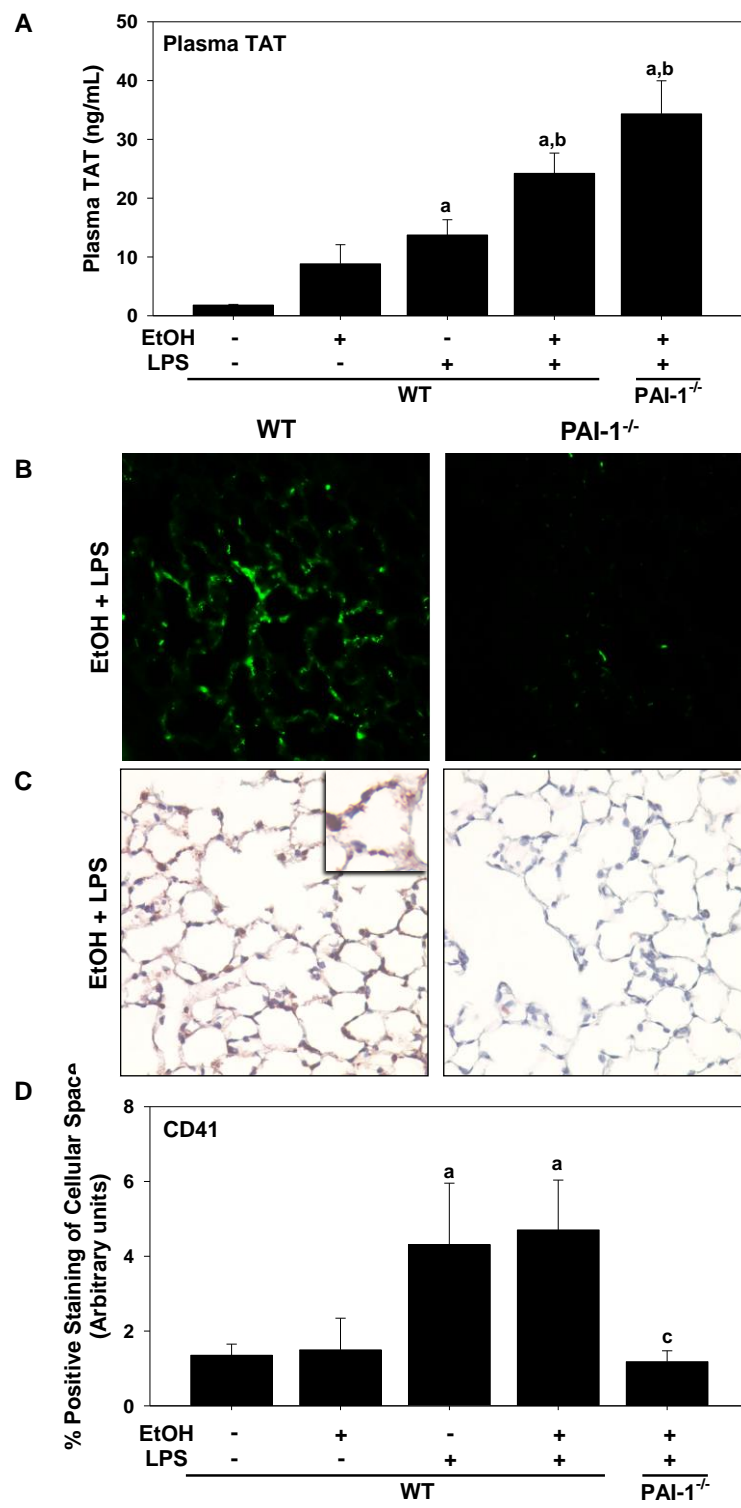
As the canonical inhibitor of urokinase-type plasminogen activator and tissue-type plasminogen activator, PAI-1 induction prevents the degradation of fibrin by plasmin. Therefore, fibrin accumulation in lung tissue was also measured. Figure 3.1B shows representative photomicrographs of lung tissue stained immunofluorescently for fibrin. LPS administration caused fibrin to accumulate in both vascular and extravascular tissue in the lung 24 h after LPS. There was no detectable effect of LPS on this variable at the 4 h time point (not shown). In contrast, ethanol feeding alone did not affect pulmonary fibrin deposition; however, it enhanced fibrin accumulation caused by LPS administration (Figure 3.1B).

2. PAI-1 deficiency blocks alcohol-enhanced pulmonary fibrin deposition and LPS-induced pulmonary platelet accumulation.

Fibrin may accumulate at sites of injury via enhanced activation of the coagulation cascade (i.e., thrombin activation), or by impaired fibrinolysis (i.e., PAI-1 induction). Therefore, the effect of PAI-1 deficiency on activation of the coagulation cascade was determined. In the current study, ethanol pre-exposure enhanced *Pai-1* expression in the lung after LPS exposure, and this enhanced *Pai-1* expression correlated with increased deposition of fibrin in lung tissue (Figure 3.1A and 3.1B). LPS administration significantly increased plasma TAT (4 h after injection) by 7-fold, indicating activation of the coagulation cascade (Figure 3.2A). Ethanol feeding alone did not significantly enhance plasma TAT; however, ethanol significantly enhanced the increase caused by LPS administration, with values ~13-fold over control. Interestingly, PAI-1 deficiency

Figure 3.2. Effect of PAI-1 deficiency on pulmonary fibrin accumulation and platelet accumulation.

(A) Plasma thrombin anti-thrombin (TAT) levels. (B) Representative photomicrographs (400x) of pulmonary fibrin deposition detected immunofluorescently in paraffin-embedded tissues 24 h after LPS injection. (C). Representative photomicrographs (400x) of platelets detected immunohistochemically in paraffin-embedded sections via integrin α_{IIb} (CD41) 4 h after LPS injection. (D) Quantitative image analysis of CD41-positive staining. Results are reported as means \pm standard error mean (SEM; n= 4-6), ^a, $p < 0.05$ compared to pair-fed control, ^b, $p < 0.05$ compared to LPS alone, ^c, $p < 0.05$ compared to WT animals. Appears in Poole LG et al. *Am J Respir Cell Mol Biol*. 2017 Apr 26. DOI: 10.1165/rcmb.2016-0184OC, reprinted with permission.



dramatically attenuated pulmonary fibrin deposition (Figure 3.2B), despite plasma TAT being unchanged in knockout animals (Figure 3.2A).

One potential mechanism by which fibrin matrices can be pro-inflammatory is by contributing to platelet aggregation. Fibrin can drive platelet aggregation at sites of injury, and in turn, the platelets themselves may propagate injury (102). Therefore, platelet accumulation in lung tissue was determined immunohistochemically by detecting the platelet-specific integrin $\alpha_{IIb}\beta_3$ and subsequently performing quantitative image analysis (Figure 3.2C and 3.2D). Ethanol feeding alone had no significant effect on platelet accumulation (CD41-positive staining), and LPS administration significantly enhanced platelet accumulation in lung tissue. LPS-induced platelet accumulation was not affected by ethanol pre-exposure. PAI-1 deficiency prevented platelet accumulation in lung tissue after LPS administration.

3. PAI-1 deficient mice are protected from alcohol-enhanced edematous lung injury caused by LPS.

The results described in Figures 3.1 and 3.2 suggest that ethanol feeding enhances LPS-induced fibrin deposition at least in part by super-inducing PAI-1 expression (Figure 3.1A and 3.2B). Additionally, Figure 3.2C indicates that PAI-1 is critical for LPS-induced platelet aggregation in lung tissue. Previous studies indicate that PAI-1 contributes to inflammatory damage to the lungs (53, 54), as well as other organs (e.g., liver) (38), and that platelet aggregation is involved in the development of ALI (103). It is also known that chronic ethanol feeding sensitizes the lung to endotoxemia-induced ALI and enhances edematous lung

injury (e.g.,(28, 104)). Therefore, the effect of PAI-1 deficiency on alcohol-enhanced experimental ALI was determined.

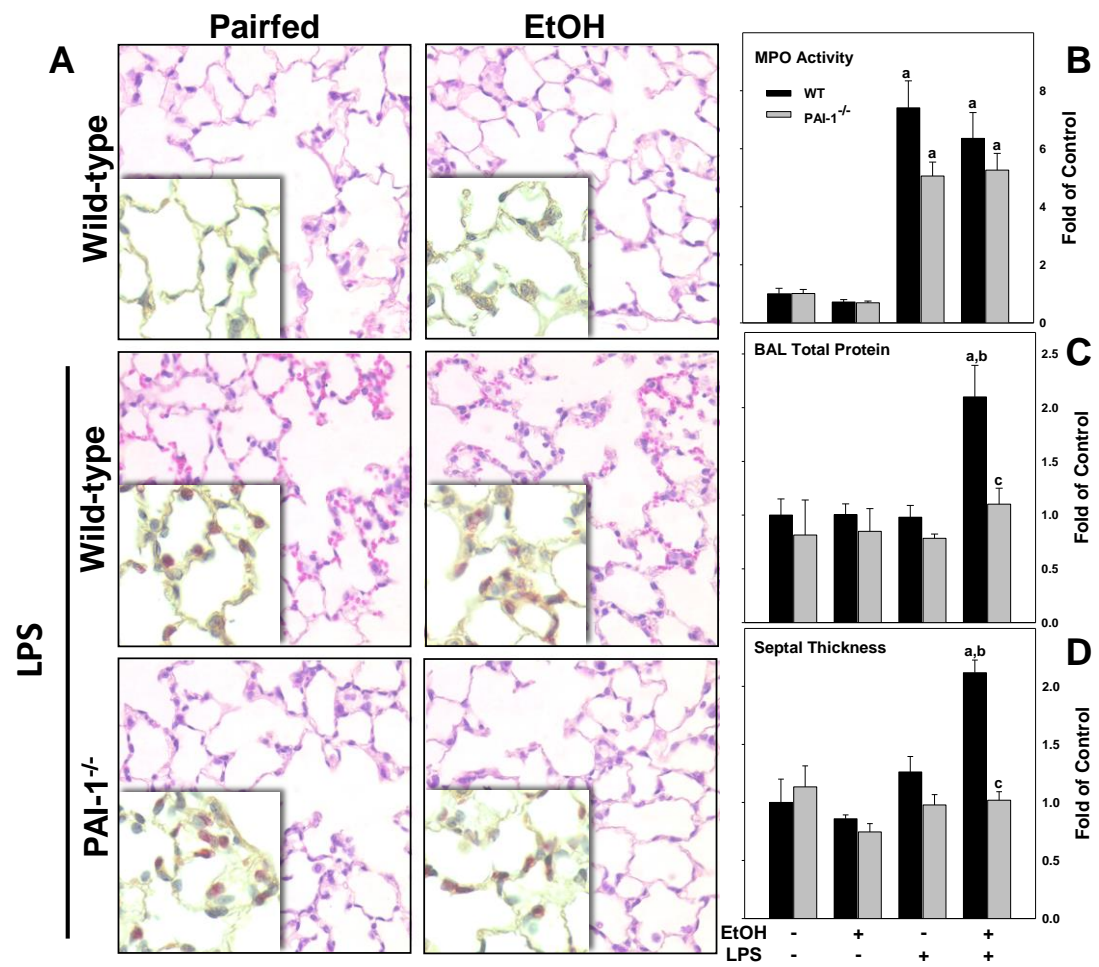
There was no significant effect of diet or genotype on growth of animals over the six week feeding period (not shown). Additionally, neither ethanol pre-exposure nor genotype affected attrition after LPS injection. As expected (104), extrathoracic LPS administration caused pulmonary injury and inflammation, as indicated by extravasation of erythrocytes (4 h after LPS, data not shown) and inflammatory cells (4 h after LPS) [Figure 3.3A (insets) and 3.3B]. At the 4 h time point, there was no discernable effect of ethanol pre-exposure on LPS-induced injury. Additionally, ethanol feeding was associated with pulmonary edema 24 hours after LPS administration, as indicated by an increase in BAL total protein content (Figure 3.3C) and enhanced thickening of the alveolar septa (Figure 3.3D). The increase in BAL protein and in septal thickening caused by the interaction of ethanol and LPS was completely attenuated in PAI-1^{-/-} mice compared to wild-type (Figure 3.3A, bottom panels, Figure 3.3C, and 3.3D). LPS administration increased the number of pulmonary neutrophils, as indicated by an increase in MPO activity (Figure 3.3B). Ethanol exposure did not significantly alter the increase in MPO activity caused by LPS.

4. Alcohol enhances LPS-induced cytokine/chemokine expression; effect of PAI-1 or integrin β_3 deficiency.

The results of the current study indicate that PAI-1 deficiency attenuates alcohol-enhanced pulmonary fibrin deposits and platelet accumulation, and also protects against alcohol-enhanced acute lung injury. Although the number of

Figure 3.3: Effect of PAI-1 deficiency on ethanol-enhanced ALI.

(A) Representative photomicrographs (400×, hematoxylin & eosin) of formalin-fixed paraffin-embedded lung tissues 24 h after LPS and CAE staining (inset, 800×). Neutrophils are shown as bright pink cells (4 h after LPS). (B) Pulmonary MPO activity 4 h after LPS. (C) BAL total protein content 24 h after LPS. (D) Quantification of alveolar septal thickening, as described in Materials and Methods, 24 h after LPS. Results are reported as means \pm standard error mean (SEM; n= 4-6), ^a, $p < 0.05$ compared to pair-fed control, ^b, $p < 0.05$ compared to LPS alone, ^c, $p < 0.05$ compared to WT animals. Appears in Poole LG et al. *Am J Respir Cell Mol Biol*. 2017 Apr 26. DOI: 10.1165/rcmb.2016-0184OC, reprinted with permission.

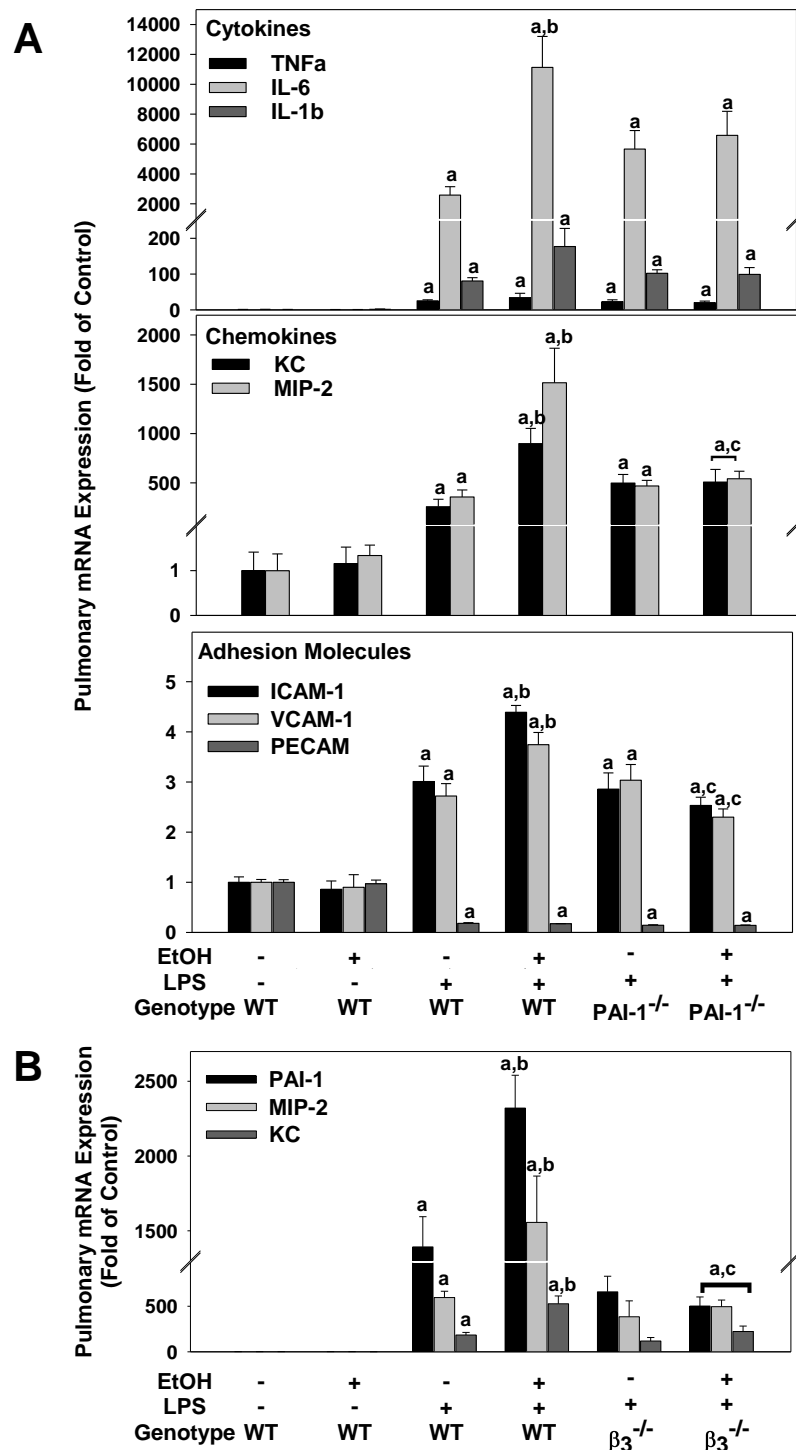


infiltrating neutrophils is unaffected by ethanol pre-exposure or PAI-1 deficiency, the phenotype of recruited inflammatory cells may be influenced by how these factors affect inflammatory cytokine and chemokine expression (105). Therefore, the mRNA expression of key inflammatory mediators was measured in whole lung homogenate (Figure 3.4). Protein levels of cytokines and chemokines relative to mRNA expression has been validated previously (104). Ethanol alone did not affect expression of any of the tested cytokines (*Tnfa*, *Il-6*, *Il-1β*; 3.4A), chemokines (KC (*Cxcl1*), MIP-2 (*Cxcl2*); 3.4A) or adhesion molecules (*Icam-1*, *Vcam-1*, *Pecam*; 3.4A) measured. LPS administration induced expression of all variables, with the exception of *Pecam*, which was significantly decreased by LPS administration (Figure 3.4A). As demonstrated previously (104), ethanol feeding enhanced the induction of *Il-6*, *Cxcl1* (KC), *Cxcl2* (MIP-2), *Icam-1*, and *Vcam-1* expression caused by LPS. With the exception of *Il-6*, these effects of ethanol were significantly attenuated in PAI-1^{-/-} mice, with values in the knockout strain similar to wild-type mice receiving LPS in the absence of ethanol feeding. For example, the expression of MIP-2 caused by ethanol/LPS was ~3-fold lower in PAI-1^{-/-} compared to wild-type mice. In the absence of ethanol pre-exposure, PAI-1 deficiency did not significantly affect LPS-induced expression of any mediators measured compared to wild-type animals (Figure 3.4A).

The CXC chemokines, MIP-2 (CXCL1) and KC (CXCL2) are murine homologues of IL-8. In experimental studies, these chemokines are critical for the development of ALI (106). In the current study, ethanol feeding enhances expression of the chemokines MIP-2 and KC, and this effect is significantly

Figure 3.4: Effect of PAI-1 deficiency and integrin β_3 deficiency on LPS-induced cytokine and chemokine expression.

(A) Pulmonary mRNA expression in whole lung homogenate of cytokines, chemokines, and vascular adhesion molecules measured by qRT-PCR in wild-type and PAI-1 knockout mice treated with ethanol and/or LPS. (B) Pulmonary mRNA expression of select chemokines measured by qRT-PCR in wild-type or integrin β_3 knockout mice measured by qRT-PCR. Results are reported as means \pm standard error mean (SEM; n= 4-6), ^a, $p < 0.05$ compared to pair-fed control, ^b, $p < 0.05$ compared to LPS alone, ^c, $p < 0.05$ compared to WT animals. Appears in Poole LG et al. *Am J Respir Cell Mol Biol*. 2017 Apr 26. DOI: 10.1165/rcmb.2016-0184OC, reprinted with permission.



attenuated in PAI-1 deficient mice (Figure 3.4A). One potential mechanism that may drive expression of these CXC chemokines is aggregation of platelets in the pulmonary vasculature (107). Results in Figure 3.2C indicate that PAI-1 deficiency attenuates LPS-induced platelet aggregation in the lung, which is mediated, at least in part, by integrin $\alpha_{IIb}\beta_3$ binding to fibrin. Therefore, the effect of β_3 integrin deficiency on ethanol-enhanced expression of the chemokines MIP-2 and KC in whole lung homogenate were determined (Figure 3.4B). The enhanced induction of pulmonary MIP-2 and KC caused by the interaction of ethanol and LPS exposure was completely attenuated by β_3 deficiency. Interestingly, the super-induction of PAI-1 caused by this interaction was also completely attenuated (Figure 3.4B). Markers of other integrin β_3 -expressing cells, such as Th17 cells (e.g., IL-22 and IL-23), were also measured, and found to be unaffected by diet or genotype (data not shown). Moreover, the surface expression of CD41 is altered by β_3 deficiency (108), so quantitation of CD41 staining via image analysis was not feasible. However, morphologic assessment of CD41 staining in β_3 knockout mice suggested that β_3 deficiency blocks LPS-induced platelet accumulation in the lung after ethanol (not shown), similar to findings in PAI-1 deficient mice (Figure 3.2).

D. Discussion

Endotoxemia in the setting of sepsis is one of the top causes of acute lung injury. As mentioned in the Introduction, chronic alcohol exposure is one of the most clinically important susceptibility factors for this deadly disorder in at-risk

individuals. The goal of the current study was to investigate the mechanisms responsible for these events in mice exposed to ethanol chronically (6 weeks) followed by induction of endotoxemia. Furthermore, this study explored the role of anti-fibrinolytic PAI-1 and β_3 integrins using genetically engineered (i.e., knockout) animals.

These studies revealed important and novel observations. First, it was observed that extrathoracic LPS induced the deposition of fibrin in the lung, which was associated with elevated PAI-1 levels (Figure 3.1). These effects were also associated with the accumulation of platelets in the lung (Figure 3.2). LPS caused inflammatory injury in the lung, as indicated by accumulation of neutrophils and extravasation of erythrocytes (Figure 3.3), and induced expression of several pro-inflammatory mediators (Figure 3.4). Second, although exposure to ethanol alone did not affect these variables, ethanol enhanced LPS-induced PAI-1 expression (Figure 3.1A), pulmonary fibrin deposition (Figure 3.1B) and plasma TAT (Figure 3.2A), and indices of transient lung damage (Figure 3.3), as well as expression of proinflammatory mediators described above (Figure 3.4). As such, these studies further strengthen available data implicating alcohol as a major susceptibility factor in acute lung injury. Third, PAI-1 deficiency dramatically blunted pulmonary fibrin deposition and platelet accumulation, as well as attenuated alcohol-enhanced ALI. PAI-1 deficiency also significantly protected against the alcohol-enhanced expression of inflammatory mediators (Figures 3.2-4). Finally, mice lacking $\alpha_{IIb}\beta_3$, the primary platelet

receptor for fibrinogen, displayed a dramatic reduction in early inflammatory change after alcohol/LPS challenge (Figure 3.4).

In the current study, alcohol enhanced the activation of the coagulation cascade, as well as induction of PAI-1 (Figures 3.1 and 3.2), both of which are capable of enhancing fibrin ECM deposition (Figure 3.1). Changes in the amount and composition of the ECM are well-recognized events in the pathology of end-stage diseases (e.g., hepatic cirrhosis and pulmonary fibrosis). However, the extracellular matrix is a dynamic and responsive entity, and subtle alterations to the ECM may be involved in inflammatory/pre-fibrotic stages of disease (43). The term “transitional tissue remodeling” describes qualitative and quantitative changes of matrix proteins in response to insults that do not alter the overall architecture of the organ. Recent studies suggest that transitional tissue remodeling contributes to damage caused/enhanced by alcohol in several organs, including the liver and the lung (44). The Arteel group has demonstrated that the hepatic fibrin ECM responds dynamically to alcohol exposure, sensitizing the liver to LPS-induced inflammatory damage (38). Similarly, chronic alcohol exposure alters the expression and degradation of the pulmonary ECM, favoring fibronectin deposition (40). The activation of the coagulation cascade is a key shared response to acute organ injury that transiently alters the ECM. Although these changes often revert without any prolonged tissue damage, they have the potential to alter the immune/inflammatory response to stress.

The finding that knocking out PAI-1 almost completely attenuated alcohol-enhanced fibrin deposition in the lung (Figure 3.2B), despite no effect on plasma

TAT (Figure 3.2A) indicates that PAI-1 induction is critical under these conditions, regardless of activation of the coagulation cascade. These results are in line with previous investigations into fibrin and PAI-1 in mouse liver (38). These results suggest that the half-life of fibrin is regulated predominantly at the level of fibrinolysis, rather than at the level of deposition. Interestingly, ethanol did not impact the increase in neutrophil recruitment caused by LPS (Figure 3.3B), but nevertheless increased injury (Figures 3.3A, 3.3C, 3.3D) and proinflammatory chemokine expression (Figure 3.4). These results suggest PAI-1/fibrin is mediating the inflammatory injury rather than inflammatory cell recruitment, *per se*. Fibrin matrices contribute to inflammation in many models through several potential mechanisms [see (101) for review]. For example, fibrin matrices provide a chemotactic substrate for monocytes and leukocytes and induce cytokine expression (109).

In addition to physiochemical effects that may enhance inflammatory injury, fibrin may facilitate recruitment of platelets to sites of injury. Indeed, the enhanced lung damage caused by the interaction between ethanol and LPS was associated with an increase in platelet recruitment and/or indices of platelet activators (i.e., TAT; Figure 4). Impairment of the ability of platelets to bind to fibrin via integrin $\alpha_{IIb}\beta_3$ (such as in the case of $\beta_3^{-/-}$ mice) also prevented injury. Platelets are known to be involved in many forms of lung injury (102), including allergic inflammation (110), cystic fibrosis (111), acid aspiration (112), and importantly for the current study, endotoxemia (113). Platelets adhere to the pulmonary endothelium after LPS injection (113). After this initial adherence,

platelets can become more tightly bound and aggregated by activation of integrin $\alpha_{IIb}\beta_3$ by binding to fibrin. Additionally, thrombin can then further activate platelets via the protease-activated receptors (PARs) (114, 115). Adhered and activated platelets can promote inflammatory injury by releasing mediators (e.g., CD40L) that upregulate expression of chemokines and vascular adhesion molecules in endothelial cells (107). In the current study, PAI-1-dependent platelet accumulation was associated with enhanced expression of the chemokines MIP-2 and KC, as well as vascular adhesion molecules *Icam-1* and *Vcam-1* (Figure 3.4); these effects were paralleled by integrin β_3 deficiency (Figure 3.4B), supporting the hypothesis that these are linked events.

Interestingly, PAI-1 appeared to play a dominant role in mediating this effect; specifically, PAI-1-deficient mice were protected against injury and platelet accumulation despite not decreasing plasma TAT levels. These effects of ethanol/LPS represents a hypothesized pathway for the accumulation of fibrin, platelet recruitment and activation, and the progression of inflammatory injury in which both PAI-1 and platelet β_3 integrins are necessary (Scheme 3.2).

PAI-1, like many acute phase proteins, can act as a “double-edged sword;” it is required for injury, and in some cases, required for wound healing and repair. For example, the Arteel group has demonstrated that PAI-1 deficient animals are protected against both acute and chronic alcohol-induced liver injury; importantly, injury in these models is inflammatory and relatively low-grade. On the other hand, PAI-1 deficient mice show exacerbated damage in more robust models of liver injury, including acetaminophen overdose (116, 117), partial

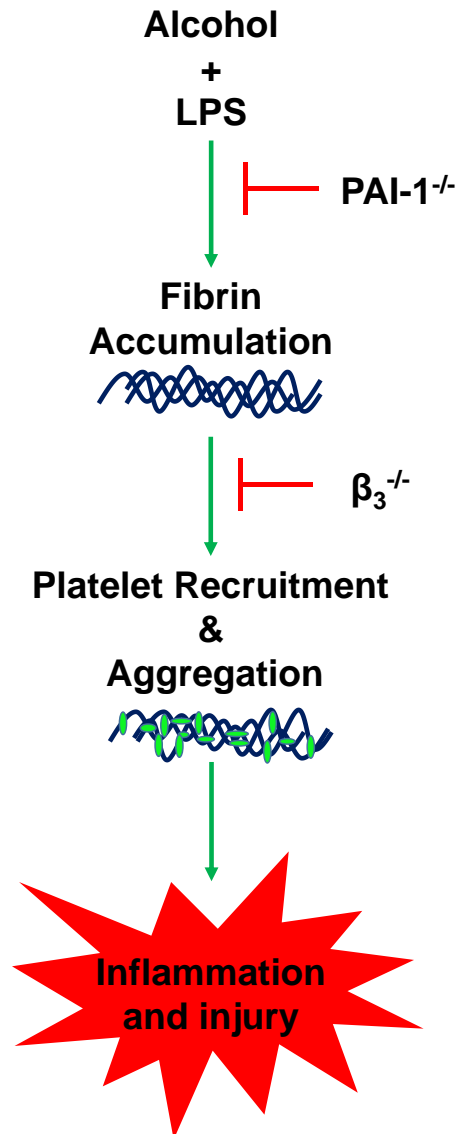
hepatectomy (118), and in carbon tetrachloride-induced fibrosis (119). Similarly, PAI-1 deficiency has been demonstrated to enhance acute lung injury in severe models of ALI/ARDS, such as intratracheal LPS instillation (120). The studies presented in this chapter demonstrate that PAI-1 deficient mice are protected against the enhancing effect of alcohol on extrathoracic endotoxemia induced acute lung injury, in which injury is less severe than the thoracic injection model. These data therefore indirectly suggest that PAI-1 may also play dual roles in lung injury, depending on the severity of that injury.

In summary, the study presented in this chapter has demonstrated a critical role for PAI-1 in experimental alcohol-enhanced acute lung injury. PAI-1-deficient mice were protected against alcohol-enhanced inflammatory lung injury. It is hypothesized that these effects are mediated, at least in part, through PAI-1-induced fibrin accumulation and subsequent platelet aggregation driven via integrin $\alpha_{IIb}\beta_3$. This study provides insight into novel targets for therapy to prevent the development and/or progression of acute lung injury in at-risk patients.

Scheme 3.2: Working hypothesis for the role of PAI-1 in alcohol-enhanced acute lung injury.

It is hypothesized that excessive accumulation of fibrin after tissue injury and/or endotoxemia (LPS) can contribute to $\alpha_{IIb}\beta_3$ -mediated platelet accumulation and aggregation. Activated platelets can contribute to acute lung injury by many potential mechanisms, including release of pro-inflammatory cytokines and chemokines from inflammatory cells and ultimately, enhanced tissue injury. These effects are dramatically exacerbated by ethanol pre-exposure. Interventions designed to block this pathway, such as PAI-1 blockade and β_3 integrin inhibition may stop the propagation of injury and promote wound healing. Appears in Poole LG et al. *Am J Respir Cell Mol Biol*. 2017 Apr 26. DOI: 10.1165/rcmb.2016-0184OC, reprinted with permission.

Working Hypothesis



CHAPTER IV

ACUTE-ON-CHRONIC ALCOHOL EXPOSURE PROMOTES PULMONARY INFLAMMATION AND AFFECTS LUNG MECHANICS

A. Introduction

The consumption of alcoholic beverages is a common practice throughout the world. In fact, it has been reported that 87.6% of adults in the United States will consume alcohol at some point (98). The detrimental health effects of excessive alcohol consumption have been well-characterized. Alcohol is the fifth leading risk factor for premature death and disability worldwide (100), contributing to the development of over 200 disease states (99). Although the liver is considered to be the major target of alcohol toxicity, alcohol also damages several distal organs. The lung is recognized as a target of chronic alcohol abuse, and alcohol-related susceptibility to lung injury is estimated to account for tens of thousands of deaths in the United States each year (18). Although chronic alcohol consumption is not directly linked to the development of lung disease per se, it appears to sensitize the lung to damage from other causes. For example, acute respiratory distress syndrome (ARDS), the most severe form of acute lung injury (ALI), occurs 3.7 times more often in people meeting the

diagnostic criteria for alcohol use disorders, and these patients have a much worse prognosis (19-21).

Although some of the clinical impacts of alcohol consumption on the lung are well-described, mechanism(s) by which ethanol impacts pulmonary function are incompletely understood. These gaps in our knowledge are due, at least in part, to the complexity of alcohol consumption in the human population. For example, the impact of alcohol consumption/abuse on the lungs is often confounded by comorbidities (e.g., smoking) (121). Furthermore, it is possible that alcohol consumption has a complicated dose response in the lung, vis-à-vis what is observed in cardiovascular disease (i.e., hormesis) (121).

Rodent models of alcohol exposure to study the effects of alcohol on the lung have been employed to compensate, at least in part, for the limitations of the human consumption data. Common models include ethanol in drinking water, acute (binge) ethanol intoxication, and chronic, *ad libitum* feeding of ethanol in the Lieber-Decarli liquid diet. These animal models have been used to elucidate many important mechanisms of pulmonary alcohol toxicity, including the development of oxidative stress (4), the induction of transitional tissue remodeling (4), and the sensitization of the lung to second injuries, (such as lipopolysaccharide exposure (28, 104), burn injury (122), or infection (123)). Alcohol consumption in most models is not sufficient to overtly change lung tissue architecture, and thereby require a second 'hit'. Specifically, rodent aversion to alcohol-containing water/diet limits the blood alcohol concentrations achievable in *ad libitum* models. By extension, these models serve as paradigms

of low/moderate alcohol consumption and do not recapitulate end organ damage associated with the drinking patterns of those with alcohol use disorders (124).

Aversion to alcohol is a well-known limitation of rodent models of alcohol exposure, not only restricted to pulmonary models. Recently, a new model of acute-on-chronic alcohol exposure was developed to study alcohol-induced liver disease (80), in which chronic dietary exposure is followed by an acute bolus gavage of alcohol. It is hypothesized that this model better recapitulates the drinking patterns of an individual with an alcohol use disorder. Importantly, hepatic pathology in this acute-on-chronic model is more similar to human alcoholic liver disease. The impact of such an alcohol exposure regimen on lung architecture and function has not been previously determined. Therefore, the goal of this study was to examine lung injury and inflammation in a well-characterized experimental model of acute-on-chronic alcohol exposure.

B. Experimental Procedures

1. Animals and Treatments

Animals were administered ethanol containing liquid diet or isocaloric control liquid diet for 10 days, then a single binge of 5 g/kg ethanol or maltose dextran as described in Chapter II Section A.3

2. Clinical Chemistry

Plasma transaminase activity (ALT and AST) were measured using commercially available kits as described in Chapter II, Section C.1.

3. Histology

Formalin-fixed paraffin-embedded liver and lung sections were stained with hematoxylin and eosin and for CAE as described in Chapter II, Sections B.1 and B.3. BAL cells were stained as described in Chapter II, Section B.7.

4. RNA and real-time RT-PCR

Messenger RNA was isolated from tissue homogenate, and gene expression of F4/80, CD68, Ly6g, CXCL1, CXCL2, CXCL15, and β -actin were measured via real-time RT-PCR as described in Chapter II, Section D.

5. Pulmonary Mechanics Measurements

Pulmonary function was assessed in mice 24 hours after ethanol binge at baseline and in response to inhaled methacholine as described in Chapter II, Section E.

6. Statistical Analysis

Results are reported as means \pm standard error mean (SEM; n= 4-6). ANOVA with Bonferroni's post-hoc test was used for determination of statistical significance among treatment groups, using SigmaPlot (version 11.0). For baseline pulmonary function measurements, a Student's T-test was used to determine significance between groups. For pulmonary function in response to inhaled methacholine, statistical significance was determined using a two-way repeated measures ANOVA. If necessary, transformation of data was used to

achieve normally distributed data before ANOVA analysis. A p -value < 0.05 was selected before the study as the level of significance. ^a, $p < 0.05$ compared to pair-fed + maltose dextran binge, ^b, $p < 0.05$ compared to ethanol-fed + maltose dextran binge.

C. Results

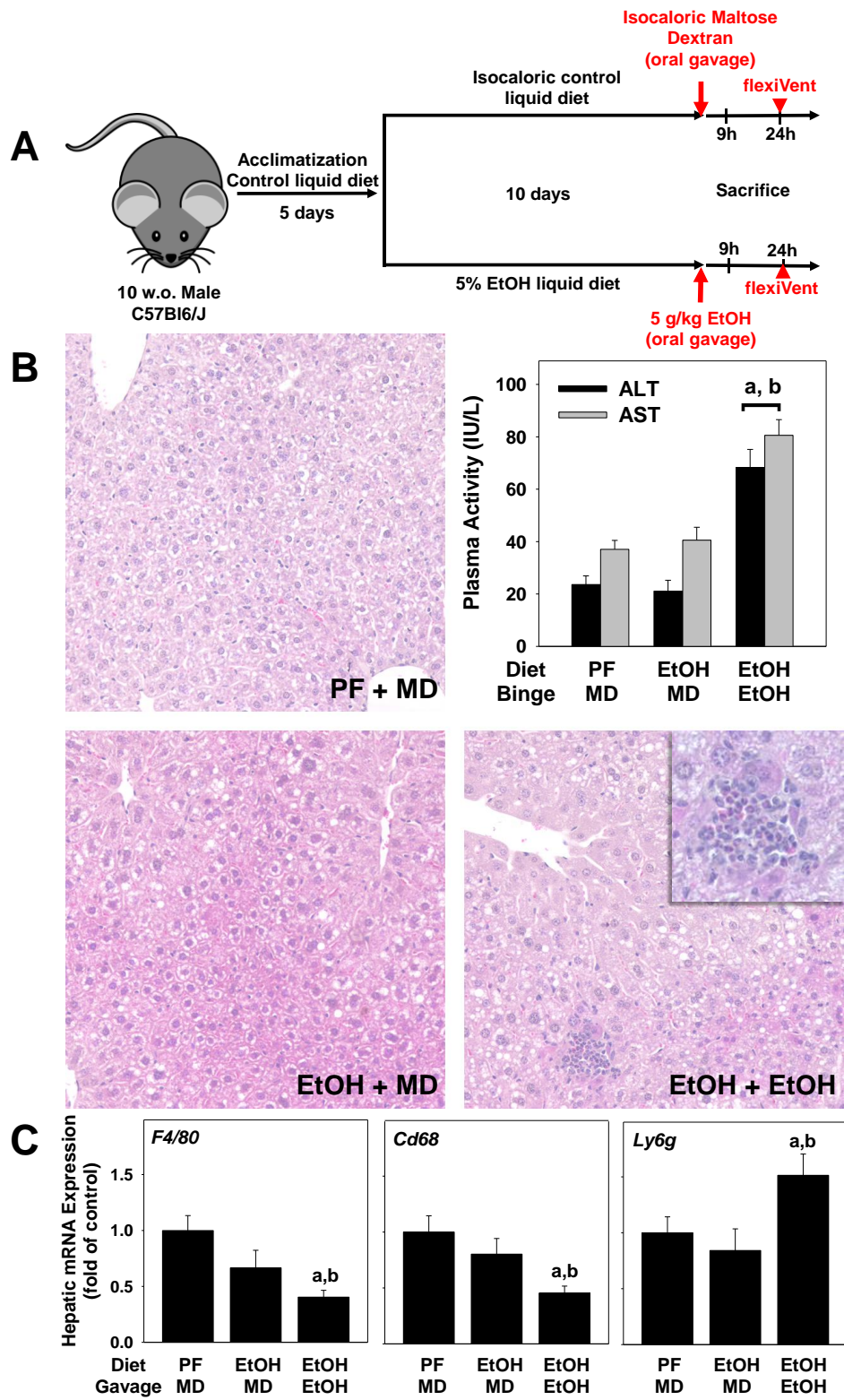
1. Alcoholic steatohepatitis results from chronic + binge ethanol exposure in mice.

Acute-on-chronic, or chronic + binge alcohol feeding produces alcoholic steatohepatitis in mice (80). Therefore, to validate the model, indices of liver injury and inflammation were measured in liver tissue 9 hours after oral gavage of ethanol or isocaloric control (Figure 4.1A). Compared to animals receiving an isocaloric control liquid diet and isocaloric control binge, animals fed an ethanol containing liquid diet developed mild hepatic steatosis, as indicated by fatty droplets in the liver tissue (Figure 4.1B, bottom left). Animals administered ethanol binge after chronic ethanol feeding (Figure 4.1B, bottom right) developed more pronounced alcoholic steatohepatitis, as indicated by fat accumulation and the development of inflammatory foci (inset). This enhanced liver injury and inflammation correlated with increased levels of plasma ALT and AST- markers of liver injury. While ethanol feeding alone did not affect plasma ALT or AST, chronic + binge ethanol exposure significantly increased ALT and AST levels, to 68 ± 7 and 81 ± 6 IU/L, respectively.

Chronic + binge ethanol feeding is known to cause immune cell infiltration in the liver, predominantly characterized by neutrophil accumulation. Therefore,

Figure 4.1. Effect of acute-on-chronic alcohol feeding on liver injury and inflammation.

(A) Scheme of chronic + binge alcohol feeding. (B) Representative photomicrographs of paraffin-embedded liver tissues stained with hematoxylin and eosin (200×) and plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, 9 h post-binge. (C) Macrophage (*F4/80* and *Cd68*) and neutrophil (*Ly6g*) markers measured in liver tissue by qRT-PCR 9 h post-binge



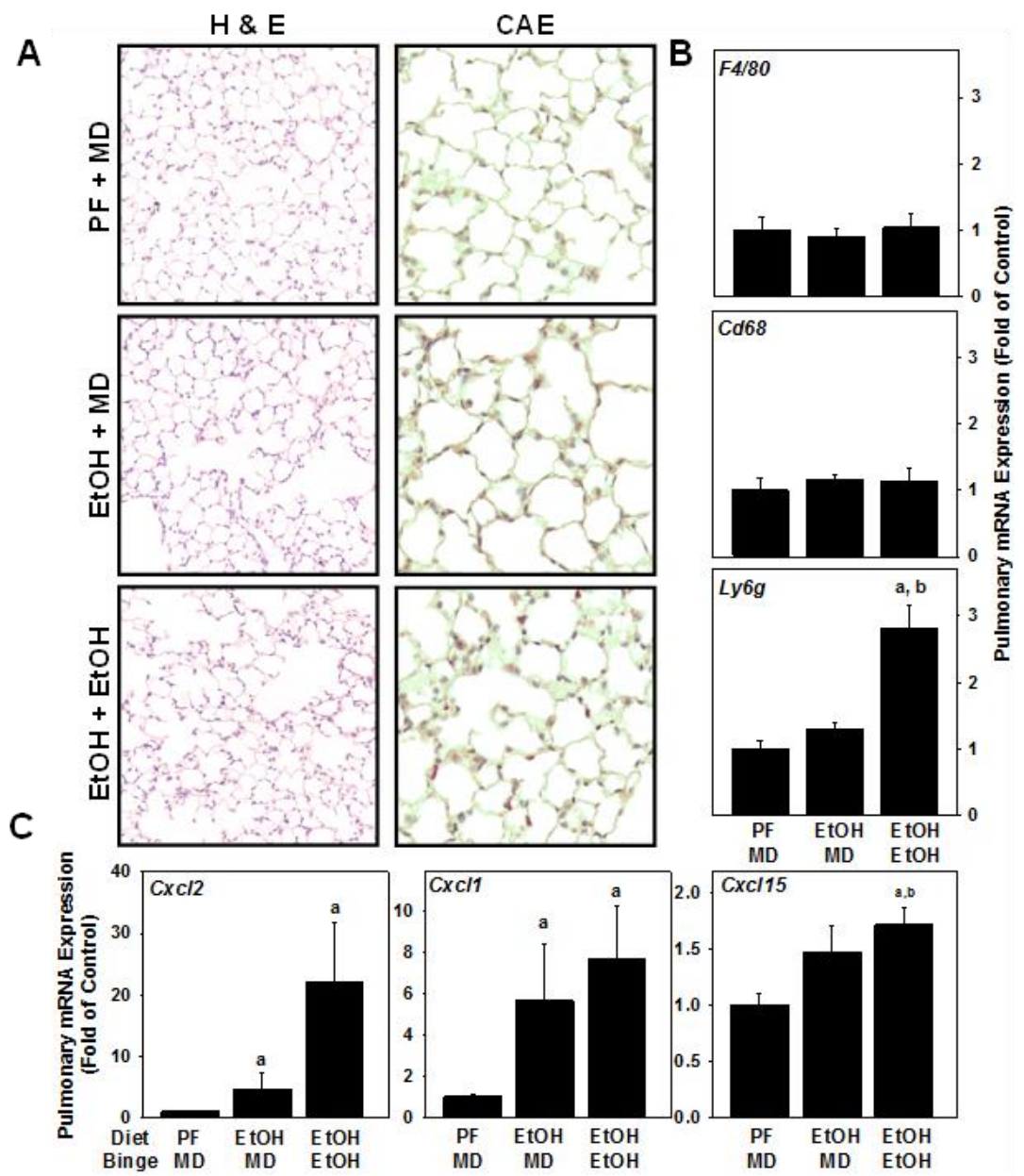
markers of macrophages (*F4/80* and *Cd68*) and neutrophils (*Ly6g*) were measured in liver tissue 9 h post-binge using qRT-PCR (Figure 4.1C). Ethanol feeding alone did not affect expression of any marker measured. However, chronic + binge ethanol significantly decreased expression of *F4/80* and *Cd68* by ~50% of control (0.4 ± 0.1 and 0.5 ± 0.2 fold of control, respectively) but significantly increased *Ly6g* expression by ~50% (1.5 ± 0.2 fold of control) suggesting increased neutrophil infiltration.

2. Chronic + binge ethanol feeding produces mild neutrophilic inflammation in the lung.

Although the effects of chronic + binge ethanol feeding have been well-characterized in the liver, the effects of this pattern of alcohol exposure on the lung are unknown. Therefore, the effects of this pattern of ethanol exposure on general lung morphology were characterized (Figure 4.2A, left). Neither ethanol-containing liquid diet alone, nor chronic + binge administration caused any overt pathological changes to the lung tissue, including the lung parenchyma and major airways (not shown). However, an increase in lung tissue cellularity was observed in lung tissue after chronic + binge alcohol exposure (Figure 4.2A, left). To determine if this was due to inflammatory cell infiltration, lung tissues were stained for chloracetate esterase (CAE), a relatively specific stain for neutrophils (Figure 4.2A, right). Ten days of ethanol feeding alone did not cause any significant neutrophil accumulation in the lung tissue; however, ethanol binge after ethanol feeding caused a marked increase in neutrophil accumulation in lung parenchymal tissue 9 h post-binge.

Figure 4.2. Lung inflammation in chronic + binge ethanol-exposed mice.

(A) Representative photomicrographs of paraffin-embedded lung tissues stained with hematoxylin and eosin (200×, left) and for chloroacetate esterase (400×, right), 9 h post-binge. CAE-positive cells are stained bright pink. (B) Macrophage (*F4/80* and *Cd68*) and neutrophil (*Ly6g*) markers measured in lung tissue by qRT-PCR 9 h post-binge. (C) Neutrophil chemokines, *Cxcl1*, *Cxcl2*, and *Cxcl15* were measured in lung tissue by qRT-PCR 9 h post-binge.



To further characterize the influx of inflammatory cells into the lung tissue, markers for macrophages and neutrophils were also measured in lung tissue via qRT-PCR (Figure 4.2B). Neither ethanol feeding alone nor chronic + binge ethanol exposure significantly affected expression of *F4/80* or *Cd68* in lung tissue. However, similarly to the effects seen in liver tissue, chronic + binge feeding significantly increased expression of the neutrophil marker *Ly6g* in lung tissue by 2.8 ± 0.3 fold of control 9 h post-binge (Figure 4.2B).

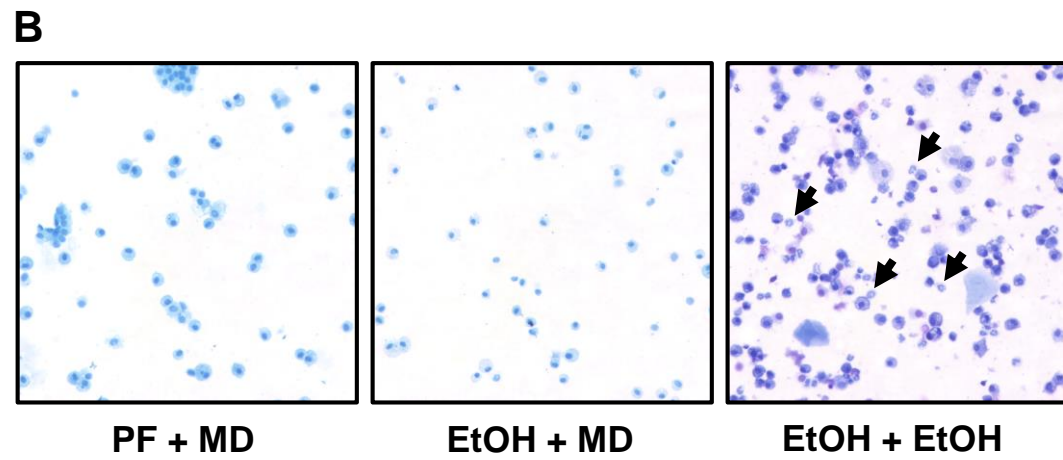
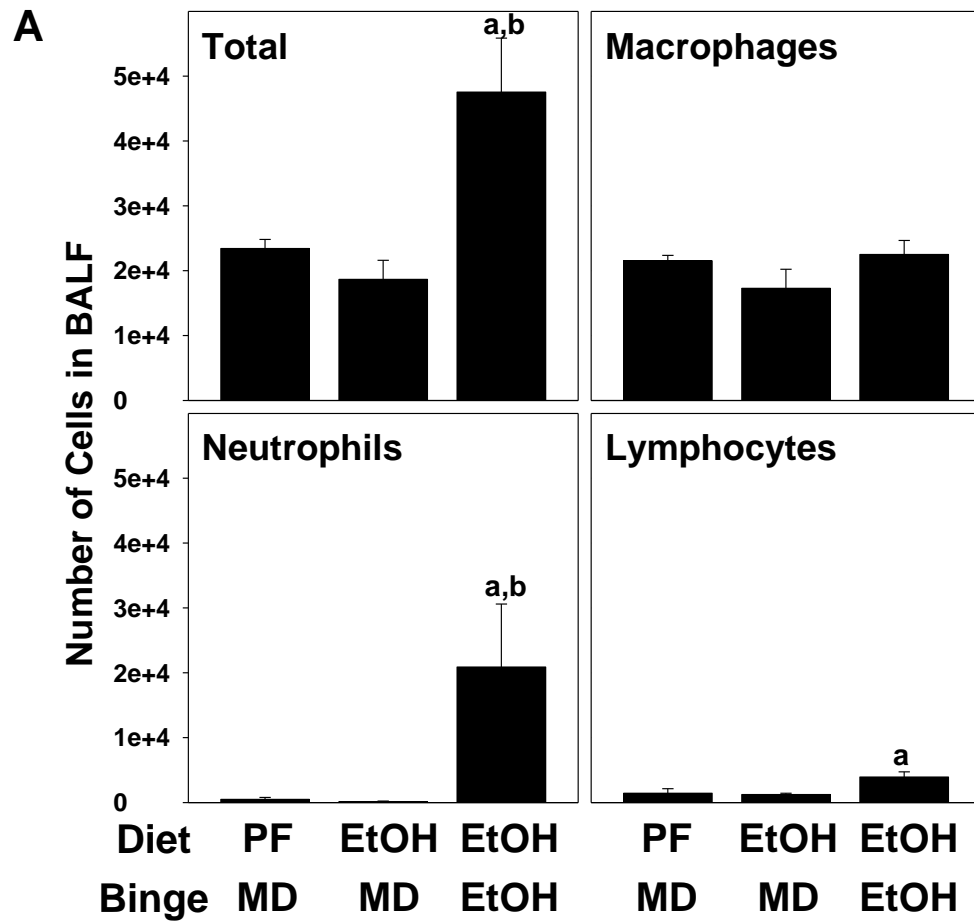
To determine whether the influx of neutrophils into lung tissue was mediated by neutrophil chemokines, the expression of *Cxcl1*, *Cxcl22*, and *Cxcl15* was measured in lung tissue 9 h post-binge (Figure 4.2C). Compared to control, animals administered 10 days of ethanol-containing liquid diet and ethanol diet plus ethanol binge had significantly elevated pulmonary expression of *Cxcl1* and *Cxcl2*. In animals exposed to chronic + binge ethanol exposure, but not 10 days of ethanol alone, expression of the lung-specific chemokine, *Cxcl15*, was increased by ~50% (1.5 ± 0.1 fold of control) of control.

3. Inflammatory cells infiltrate the airways after chronic + binge ethanol feeding.

To determine if the influx of neutrophils into the lung tissue was persistent, the number and type of cells in the bronchoalveolar lavage fluid (BALF) 24 hours after binge were examined. In control animals and animals fed ethanol-containing liquid diet alone, the total number of cells recovered was similar at approximately 20,000 cells ($23,428 \pm 1407$ and $18,666 \pm 2942$ cells, respectively). Additionally, these cells were primarily macrophages (Figures 4.3A and 4.3B). However, the

Figure 4.3. BAL cell accumulation after chronic + binge ethanol exposure.

(A) Total and differential counts of cells recovered (macrophages, neutrophils, and lymphocytes) in bronchoalveolar lavage fluid. (B) Representative photomicrographs (400x) of cytopspins stained with Kwik-Diff differential staining 24 h after binge.



number of cells recovered in animals exposed to ethanol diet and ethanol binge was significantly increased by approximately 2 fold ($47,545 \pm 8324$ cells) (Figure 4.3A). This increase in total cell number was driven primarily by increased neutrophils. Indeed, the total number of neutrophils (Figure 4.3B, arrows), as well as the number of lymphocytes, was significantly increased in these animals while the number of macrophages was unaffected (Figure 4.3A).

4. Chronic + binge alcohol exposure alters pulmonary mechanics: airway hyper-responsiveness to methacholine.

To assess the impact of chronic + binge ethanol exposure on pulmonary function, pulmonary mechanics were measured using the flexiVent system, as described in Chapter II Section E, using the forced oscillation technique. Chronic + binge ethanol exposure had no significant effect on any variables measured at baseline, including resistance (Rrs), compliance (C), pressure-volume loop (Area), Newtonian resistance (Rn), tissue damping (G), or tissue elastance (H) (Figure 4.4). However, animals exposed to chronic + binge ethanol feeding demonstrated airway hyper-reactivity in response to the inhaled bronchoconstrictive agent methacholine, as exhibited by significantly increased airway resistance (Rrs) at 25 and 50 mg/mL methacholine, and increased Newtonian Resistance, which is related to resistance in the central airways, at the same doses (Figure 4.5). Compliance, tissue damping, and tissue elastance were not significantly affected by chronic + binge ethanol exposure. Respiratory resistance (i.e., the real part of respiratory system impedance) was significantly

Figure 4.4: Effect of chronic + binge ethanol exposure on baseline pulmonary mechanics.

Baseline pulmonary mechanics assessed 24 h post-binge using the forced oscillation technique at a single perturbation frequency (2.5 Hz), or broadband perturbation (1 to 20.5 Hz), and area of pressure-volume loops.

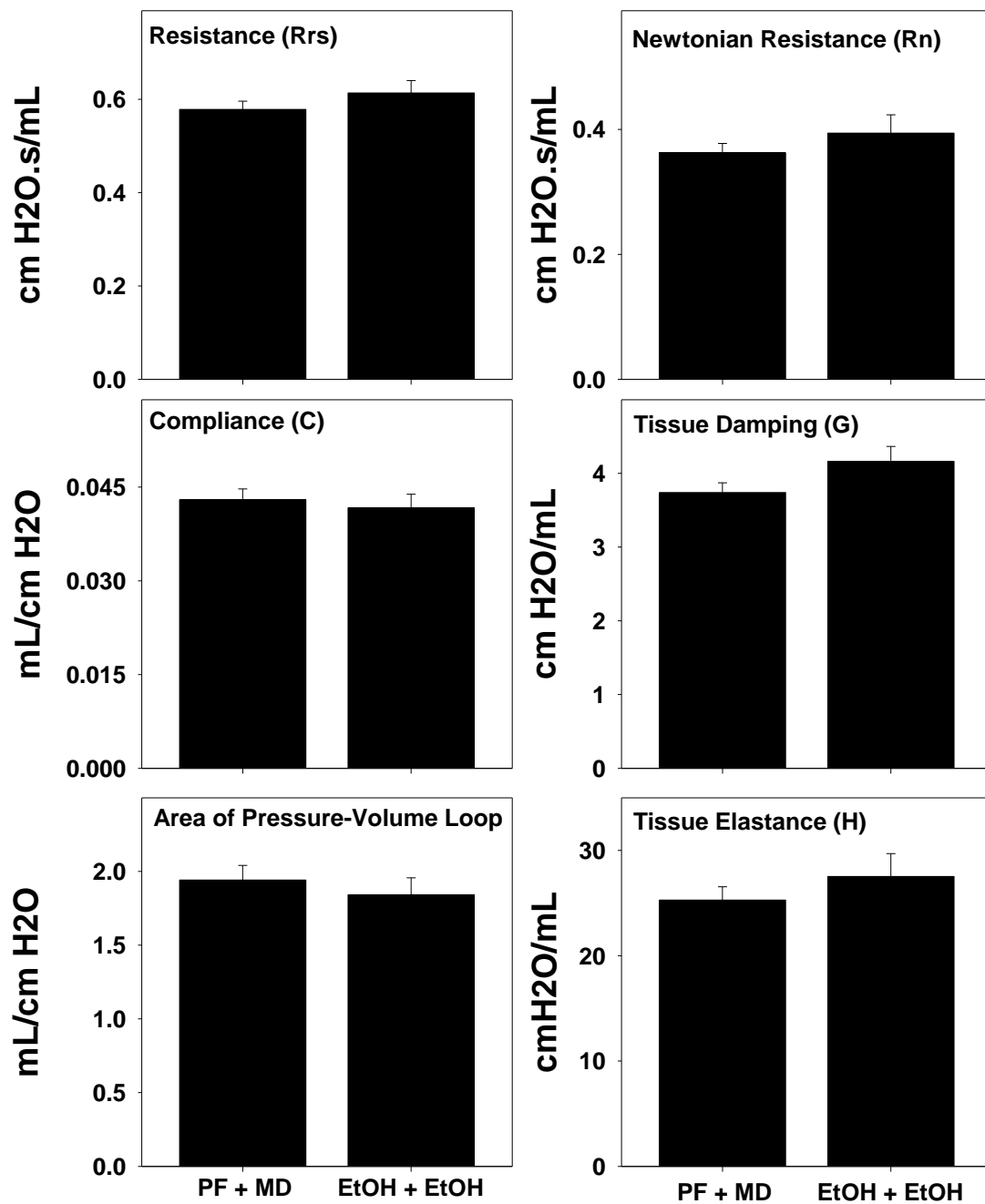


Figure 4.5: Airway hyper-responsiveness to methacholine in chronic + binge ethanol-exposed animals.

Airway reactivity to inhaled methacholine (0-50 mg/mL) assessed 24 h post-binge. Pulmonary mechanics were measured using the forced oscillation technique at a single perturbation frequency (2.5 Hz), or broadband perturbation (1 to 20.5 Hz).

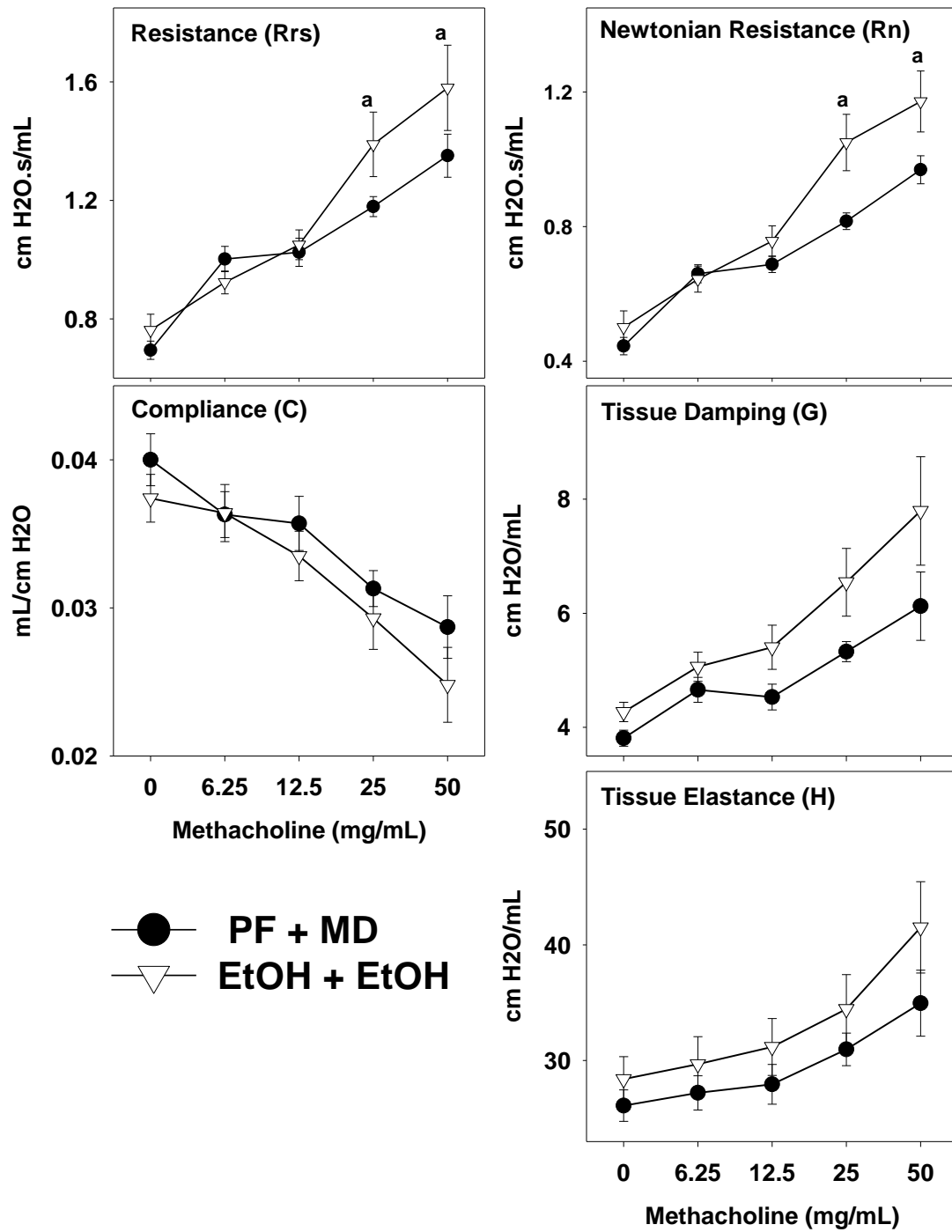
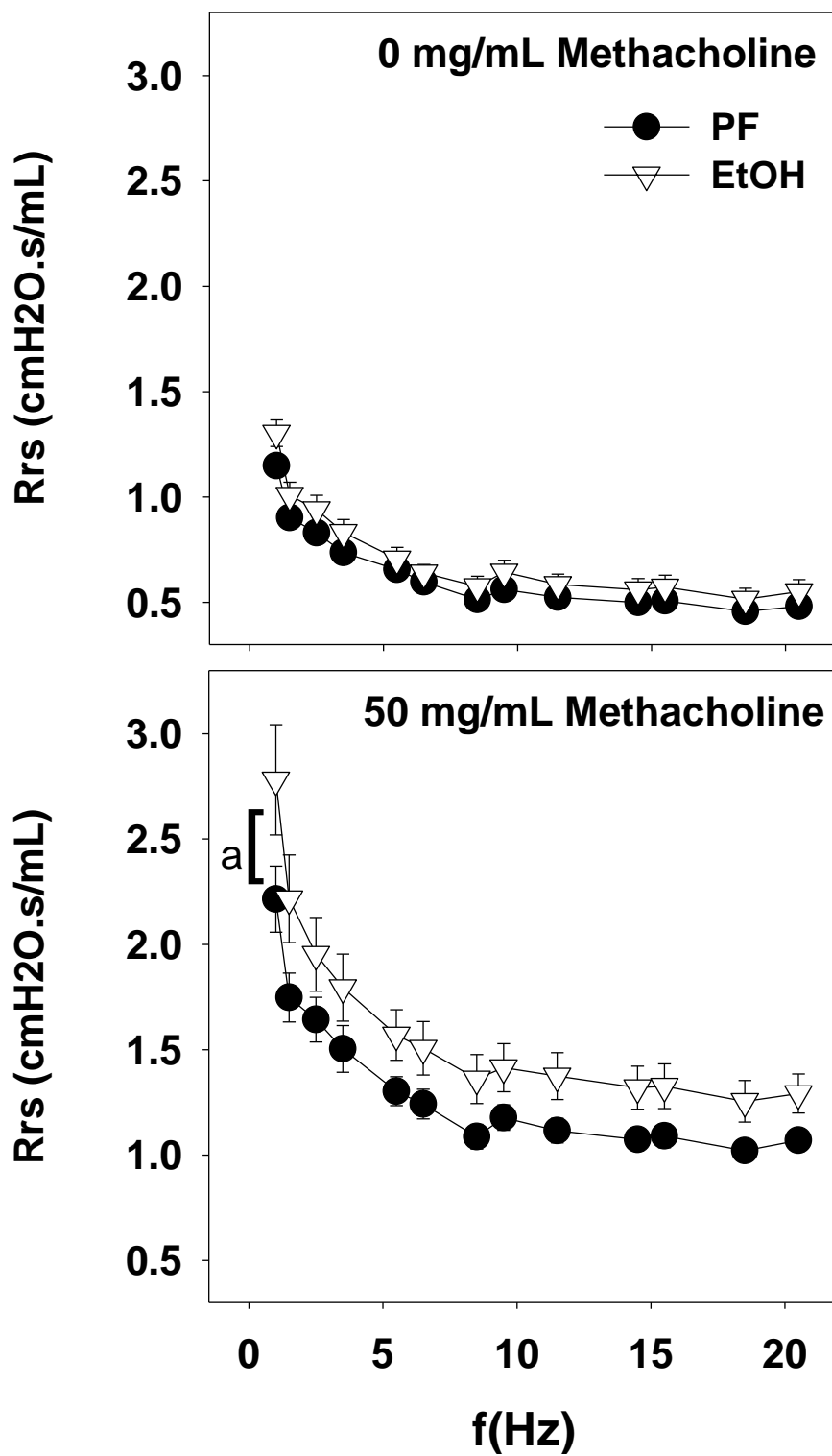


Figure 4.6: Effect of chronic + binge alcohol exposure on respiratory resistance.

Respiratory resistance (the real part of respiratory system impedance) as a function of frequency, measured 24 h post-binge in response to inhaled methacholine (0 or 50 mg/mL).



elevated at all frequencies measured, further indicating increased resistance in the central airways (Figure 4.6).

5. Discussion

Alcohol consumption is well known to negatively impact the lung and to increase the risk of upper respiratory tract infections and pneumonia (125, 126). Furthermore, individuals with a history of an alcohol use disorder are more susceptible to the development of ARDS, and often have a worse prognosis (19). Importantly, all of these pulmonary complications of alcohol consumption appear to be relegated to relatively heavy consumption. For example, the risk of ARDS only increases dramatically in individuals who drink >3 drinks per day (127). Likewise, the risk of pneumonia increases predominantly with heavy alcohol consumption (126). There are still significant gaps in our understanding on the mechanisms by which alcohol abuse mediates these effects on the pulmonary system. Nevertheless, the prevailing hypothesis is that alcohol enhances injury caused by a secondary insult (e.g., infection), rather than directly injuring the lung, per se.

As mentioned in the Introduction, discovery of mechanisms of alcohol-induced organ damage has been hampered by a lack of relevant rodent models. For example, Best et al. (73) incorrectly concluded that alcohol was not a direct hepatotoxicant, based on studies of rodents consuming alcohol-containing drinking water. Even with the development of liquid diets that increased daily alcohol consumption (e.g., Lieber-DiCarli diet) (128), histologic changes to more sensitive organs, such as the liver, did not recapitulate the human disease (124).

This factor is likely due, at least in part, by a failure of these models to achieve blood alcohol concentrations that are relevant to humans with an alcohol use disorder (129, 130). For example, blood alcohol levels in mice on the chronic (6 week) Lieber-DeCarli liquid diet model typically reach maximally ~150 mg/dL (80), and blood alcohol levels in mice on the ethanol in drinking water model are typically lower (131). As mentioned previously, even though these relatively low blood alcohol levels produce few pathologic changes in the lung, they do sensitize the lung to further injury (104).

The purpose of the current study was to explore the impact of alcohol exposure on lung histology and function in a newly developed model of chronic + binge alcohol consumption. The rationale for this study is that the blood alcohol contents (BACs) and alcohol exposure regimen may better recapitulate the pattern of alcohol consumption observed in humans (124). In the United States, the incidence of heavy binge drinking has significantly increased over the last 10 years, with currently 33% of adults reporting binge drinking in the last year. Furthermore, individuals who heavily binge are more likely to go to the emergency room with alcohol-related traumatic injuries (132). These statistics speak to the need for a relevant animal model of heavy alcohol exposure for these individuals at risk for ALI, such as the one used in the current study. As has been shown previously, this model produced significant steatohepatitis, as indicated histologically and by elevated plasma ALT and AST (Figure 4.1). Interestingly, this ethanol exposure regimen increased recruitment of neutrophils to the lung 9 hours after the ethanol binge (Figure 4.2); this pattern is similar to

what is observed in the liver (Figure 4.1). Neutrophil recruitment 9 hours after binge was associated with significant elevations in mRNA expression of the chemokines *Cxcl1*, *Cxcl2*, and *Cxcl15*. This model also increased the total number of cells collected in the BALF, driven primarily by an increase in the number of BAL neutrophils (Figure 4.3). Finally, chronic + binge ethanol exposure caused airway hyper-responsiveness to methacholine, indicative of increased resistance in the central airways (Figures 4.5-6).

Previous studies have shown that alcohol exposure is sufficient to produce pathologic changes to the lung, including ECM remodeling, oxidative stress and alveolar macrophage dysfunction (4, 43). However, these changes have previously been considered insufficient to directly cause histologic damage to the lung. Moreover, few (if any) studies have shown that ethanol exposure alone will directly induce an inflammatory response in the lung. Indeed, a similar absence of pathology was observed here when dietary ethanol was given alone (i.e., without the binge). The finding in the current study that ethanol exposure is sufficient to cause a marked increase in inflammatory cell recruitment to the lung and into the BALF is therefore novel.

Although chronic + binge ethanol feeding caused influx of inflammatory cells into the lung tissue and BAL, this inflammation was relatively mild. The functional consequences of these changes were therefore unclear. Indeed, due to the “interdependent” nature of the lung parenchyma and central airways, inflammatory damage to the parenchyma may manifest as resistance in the central airways, and vice-versa (133). This ethanol exposure regimen did not

impact any basal index of airway function, as determined by pulmonary mechanics assessment (Figure 4.4); however, it did increase airway resistance (Rrs) and Newtonian resistance (Rn) in response to methacholine (Figure 4.4). These data, combined with an upward shift in Rrs over increasing frequency (the real part of respiratory impedance), indicate increased resistance in both the central airway and lung parenchyma in response to an exogenous stimulus. These effects of alcohol exposure on pulmonary function are novel in an experimental model. However, they are not without precedence in human alcohol exposure studies. For example, airway hyper-responsiveness or “alcohol-induced asthma” has been demonstrated in humans; this phenomenon is hypothesized to be potentially the result of acetaldehyde toxicity and/or of impurities in the beverage (121, 134, 135). In contrast, others have shown that ethanol can cause bronchodilation in humans (136, 137) and it was used historically as a rescue therapy for asthma. The underlying causes of these apparent differing responses are unclear; however, alcohol dose may be critical. Furthermore, most studies have investigated the effect of concomitant alcohol exposure on lung hyper-responsiveness (138). In contrast, the impact of ethanol pre-exposure on stimulated lung mechanics, such as performed here, has not been tested. Several studies in lung and in other organs have demonstrated that alcohol has differing, even contradictory, responses depending on the timing of the exposure. Nevertheless, the development of this mouse model yields a new tool to directly determine impacts and mechanisms of alcohol on lung mechanics.

In conclusion, current mouse models of alcohol exposure have not previously been shown to cause overt inflammatory changes in the lung. One common limitation of ad libitum ethanol exposure models (e.g., ethanol in drinking water or chronic Lieber-DeCarli liquid diet) is their inability to sufficiently recapitulate risky human drinking patterns, as well as pathology on sensitive target organs, such as the liver. This liver pathology may be an important feature for reproducing the disease state of alcoholic patients at risk for developing ARDS. In fact, mortality in ARDS is almost 100% in patients with end-stage liver disease. The newly developed chronic + binge model of alcohol exposure more accurately reproduces risky drinking patterns in alcoholic patients, who are most at risk for developing sepsis-induced ARDS. The current study provides novel findings that this pattern of alcohol exposure, in the absence of any secondary inflammatory insult, caused mild neutrophilic inflammation 9 and 24 hours after ethanol binge, as well as exacerbated central airway resistance after methacholine inhalation. This animal model may be useful, and potentially more relevant, for identifying mechanisms by which alcohol abuse sensitizes at-risk individuals to ALI and ARDS.

CHAPTER V

ASSESSING THE ROLE OF HEPATIC-DERIVED CYTOKINES IN ALCOHOL- ENHANCED ALI; A NOVEL, NANOPARTICLE-BASED APPROACH TO SELECTIVELY GENETICALLY MODIFY HEPATIC MACROPHAGES

A. Introduction

Human diseases are generally multi-stage, multi-hit processes; it is therefore not surprising that multiple cells within a target organ contribute to disease pathology. The potential contribution of signals outside the target organ to disease pathology is also a well-accepted concept. However, experimental validation of these concepts has been technically difficult in some cases. The advance of temporal and/or locational control of gene expression (e.g., with conditional transgenics) has further enabled research to be performed on a system level. These advances coevolved with the era of 'omics research in which large amounts of data can be simultaneously analyzed for trends and effects. The net result is that system level analyses of disease, and organ-organ interactions are gaining attention of the research community. Mechanistic

understanding of some of these inter-organ interactions is subsequently very strong [e.g., the 'gut-liver axis' (139)].

Although less well-characterized, several studies indicate interdependence between liver and lung, potentially via mediators released from the gut. For example, mortality in ARDS patients with hepatic failure is almost 100% (140). Furthermore, pulmonary injury induced by systemic endotoxin can be altered by mediators released from the liver (e.g., TNF α) (141, 142). In an elegant study, Siore et al. (85) demonstrated that LPS-induced lung damage requires perfusion through the liver. Recently, depletion of systemic TNF α (etanercept) was demonstrated to prevent pulmonary injury in a mouse model of alcohol-enhanced ALI (35). However, while experimental data indicate that the liver is a major source of circulating TNF α after LPS administration, blocking systemic TNF α using drugs such as etanercept does not directly address the hypothesis that hepatic-derived cytokines drive injury in distal organs, such as the lungs (Figure 5.1A).

To more empirically ascertain the role of hepatic-derived cytokines in injury in other organs, including the lung, a liver-specific, targeted approach must be developed. Several "liver-specific" knockout animal models have been established using Cre-lox technology, in which the bacterial enzyme Cre (cyclization recombinase) excises a gene of interest flanked by loxp (locus of X-over in P1) sites. Cre recombinase can be engineered to be driven by specific promoters to target various cell types in the liver, such as the albumin-Cre mouse to target hepatocytes and the lecithin-retinol acyltransferase (Lrat)-Cre mouse,

among others, to target hepatic stellate cells (143). However, hepatic macrophages, Kupffer cells, are the primary source of cytokines, including TNF α in the setting of inflammatory injury. Therefore, these available liver-specific knockout mice will be insufficient to target expression of hepatic-derived cytokines. To date, the most “selective” genetic approaches available to target Kupffer cells target cells of the myeloid lineage, including M lysosome (LysM-Cre mouse) and colony stimulating factor-1 receptor (CSF1R-Cre mouse). These approaches would induce Cre-mediated excision, therefore, in all macrophage populations. There are more traditional approaches available to ablate Kupffer cells, such as administration of gadolinium chloride or liposome-encapsulated chlodronate. However, these compounds only serve to eliminate macrophages, and therefore do not allow for investigation of specific macrophage-derived mediators. Moreover, these approaches are not entirely selective to liver macrophages. For example, intravenous administration of chlodronate liposomes also targets a wide variety of macrophage populations, and is not specific to the liver (144). Taken together, these limitations highlight the need for a selective, inducible, transgenic approach.

A second variety of Cre recombinase is that which can be temporally induced by drugs. For example, the estrogen receptor agonist, tamoxifen, can be used to induce Cre expression. The commercially available R26CreER mouse utilizes an endogenous, tamoxifen-inducible promotor, Gt(ROSA)26Sor, that will, upon activation, express Cre in any cell that has been targeted by tamoxifen. Therefore, it is hypothesized that liver-targeted delivery of tamoxifen may be

sufficient to induce selective Cre-mediated excision in Kupffer cells. To achieve this targeted delivery, a nanoparticle-based approach may be utilized. Poly (lactic-co-glycolic acid) (PLGA) nanoparticles are non-toxic, biodegradable nanoparticles which, in their unmodified state, have been shown to accumulate in the liver with a majority of particles accumulating in Kupffer cells (89). The goal of the study presented in this chapter is therefore to determine if tamoxifen-loaded PLGA nanoparticles may be utilized to selectively induce Cre-mediated excision in the liver, while avoiding other macrophage populations such as those in the lung. Development of such an inducible transgenic system would be useful for determining the role of hepatic-derived cytokines in propagating injury in other organs.

B. Experimental Procedures

1. Generation of transgenic animal lines

R26CreER/mTmG mice were generated as described in Chapter II

2. Animals and treatments

Mice were injected intraperitoneally with tamoxifen, or injected intravenously or intrasplenically with tamoxifen-loaded PLGA nanoparticles as described in Chapter II, Sections 5, 6, and 7, respectively. Tissues were collected upon sacrifice as described in Chapter II.

3. Synthesis of PLGA nanoparticles

Nanoparticles were synthesized as described in Chapter II Section F.

4. Characterization of PLGA nanoparticles

PLGA nanoparticles were characterized as described in Chapter II Section G.

5. Imaging of native fluorescence

Tissues were fixed and native fluorescence of tdTomato and eGFP were imaged in liver and lung sections as described in Chapter II, Section B.6.

6. Histology

Immunohistochemistry for GFP was performed in formalin-fixed, paraffin-embedded liver and lung sections as described in Chapter II Section B.4

7. RNA isolation and qRT-PCR

RNA was isolated from liver, lung, kidney, and spleen homogenates and reverse transcribed as described in Chapter II. Messenger RNA expression of select genes was measured via qRT-PCR as described in Chapter II.

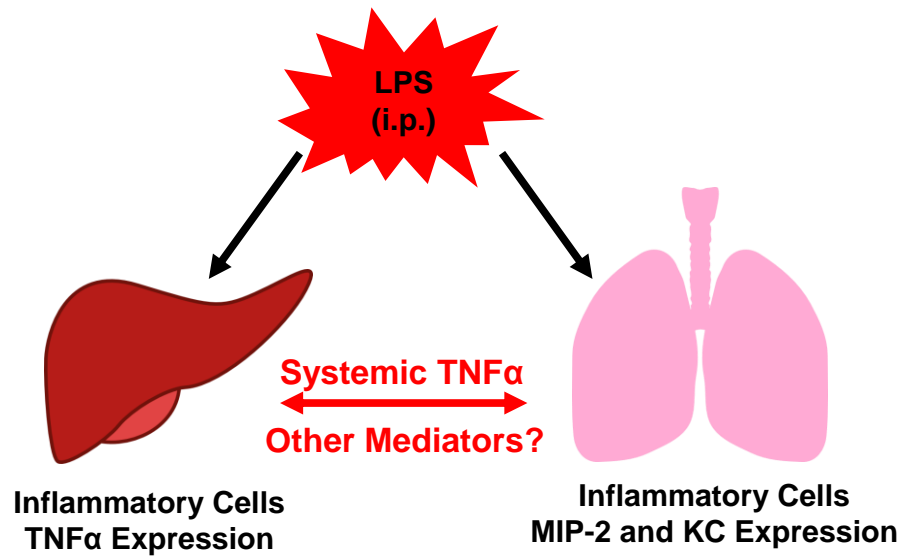
8. Statistical analysis

Statistical significance was determined as described in Chapter II, Section H. ^a, $p < 0.05$ compared to vehicle control.

Figure 5.1: Proposal to utilize transgenic reporter mouse model to investigate interactions between the liver and lung.

(A) In the setting of inflammatory injury (e.g., intraperitoneal LPS), the liver and lung may be directly damaged. However, hepatic-derived mediators, such as TNF α , may contribute to injury in the lung. (B). Scheme of tamoxifen-inducible Cre-mediated excision of the mT/mG reporter construct.

A



B

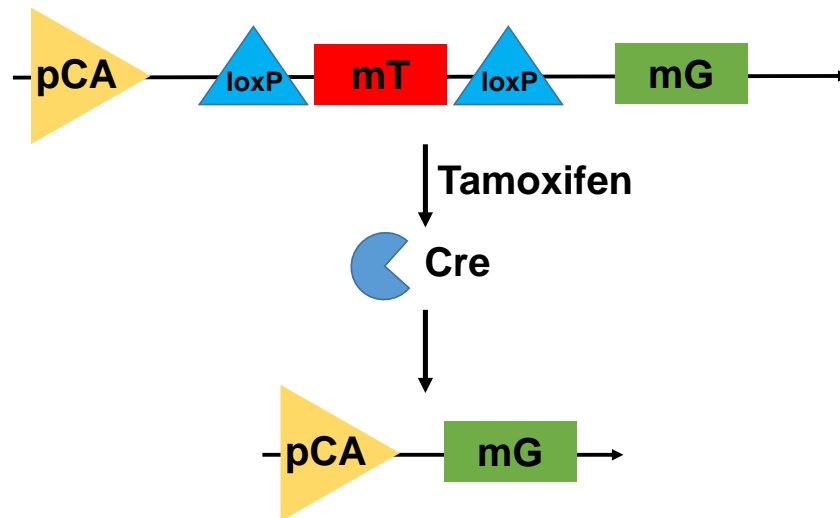
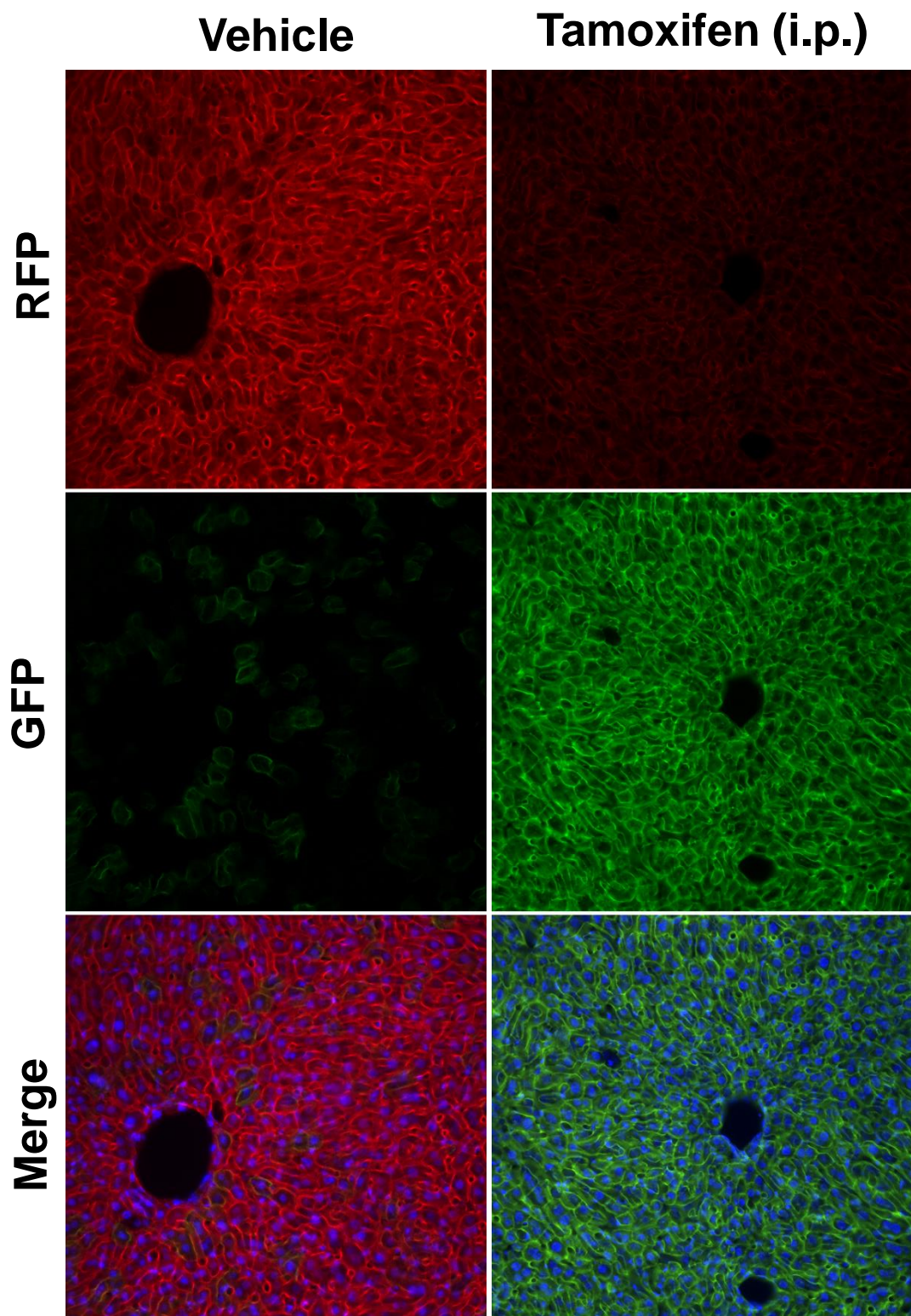


Figure 5.2: Intraperitoneal administration of tamoxifen successfully induces complete Cre-mediated excision of red fluorescence and expression of green fluorescent protein.

Representative photomicrographs (200x) of native red fluorescence (top panels) and green fluorescence (middle panels) counterstained with DAPI (blue) in frozen liver tissues after i.p. administration of tamoxifen (right) or corn oil vehicle (left) as described in Materials and Methods. Merged images are shown in bottom panels.



C. Results

1. Validation of the R26CreER⁺/mTmG⁺ mouse strain.

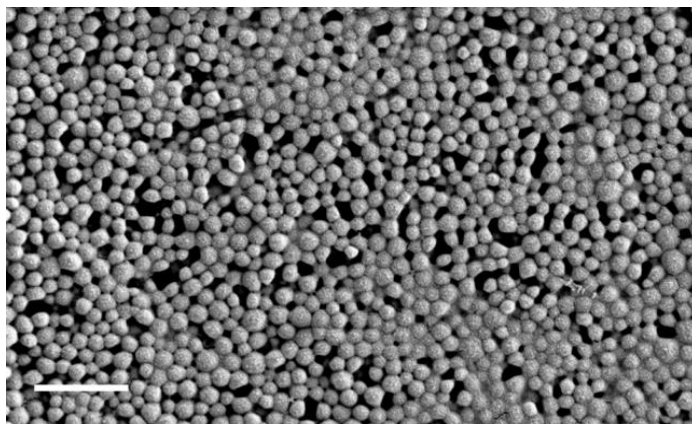
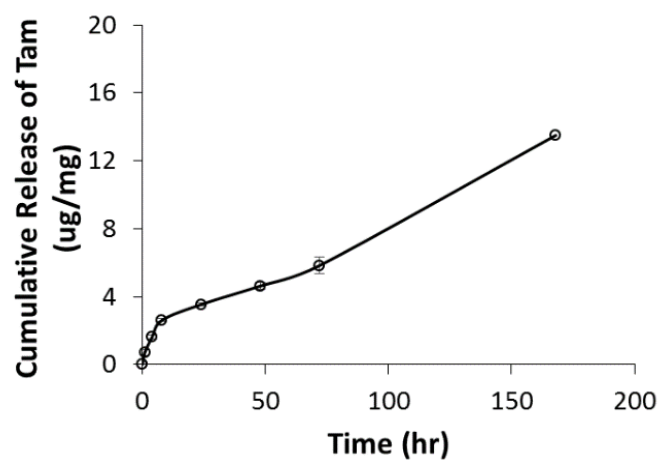
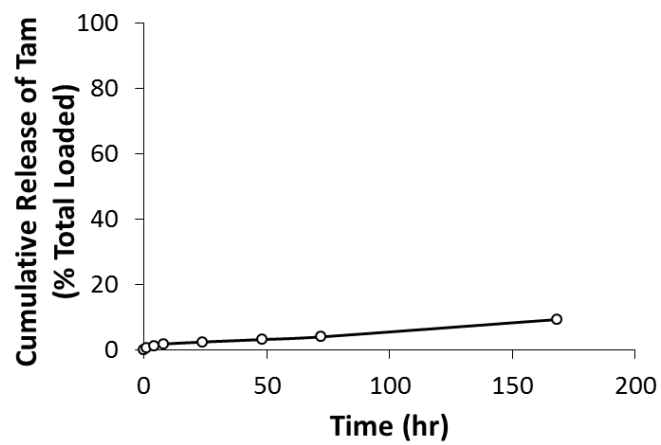
As mentioned in the Introduction, the goal of the current study was to design and characterize a transgenic animal model to selectively genetically alter hepatic resident macrophages (Kupffer cells) without transducing alveolar macrophages. A previously-established reporter mouse strain, known as the mT/mG mouse, was utilized to test this hypothesis. Prior to Cre-mediated recombination, this mouse ubiquitously expresses red fluorescent membrane-targeted tandem dimer Tomato (mT) driven by a chicken β -actin core promotor with CMV enhancer (pCA). In the presence of Cre recombinase, flox-flanked mT is excised, and the mice express membrane-targeted enhanced green fluorescent protein (mG) (Figure 5.1B). As discussed in Chapter II (Experimental Procedures), these mice were crossed with tamoxifen-inducible Cre recombinase (R26CreER). When injected intraperitoneally with vehicle (corn oil) tissues ubiquitously express mT (red fluorescence), as demonstrated by imaging native fluorescence in liver tissues. After i.p. tamoxifen injection, red fluorescence is diminished, and all cells express mG (green fluorescence) (Figure 5.2).

2. Characterization of tamoxifen-loaded PLGA nanoparticles.

PLGA nanoparticles had an average diameter of 178 ± 34 nm, and demonstrated smooth and spherical morphologies (Figure 5.3A). PLGA nanoparticles encapsulated 144.6 ± 18 μ g tamoxifen per mg of nanoparticles, corresponding to an encapsulation efficiency of 72%. The total cumulative release of tamoxifen from PLGA NPs was measured over 1 week (Figure 5.3B

Figure 5.3: Characterization of tamoxifen-loaded PLGA nanoparticles.

(A) Scanning electron microscopy (SEM) image of PLGA nanoparticles. Scale bar represents 1 μM . (B) Cumulative release of tamoxifen from nanoparticles over one week as μg of tamoxifen released per mg of nanoparticles and (C) as the percent of total tamoxifen loaded.

A**B****C**

and 5.3C). Release of tamoxifen was relatively low, with less than 20% of loaded tamoxifen released into solution after 1 week.

3. Intravenous administration of tamoxifen-loaded PLGA NP fails to selectively transduce liver cells.

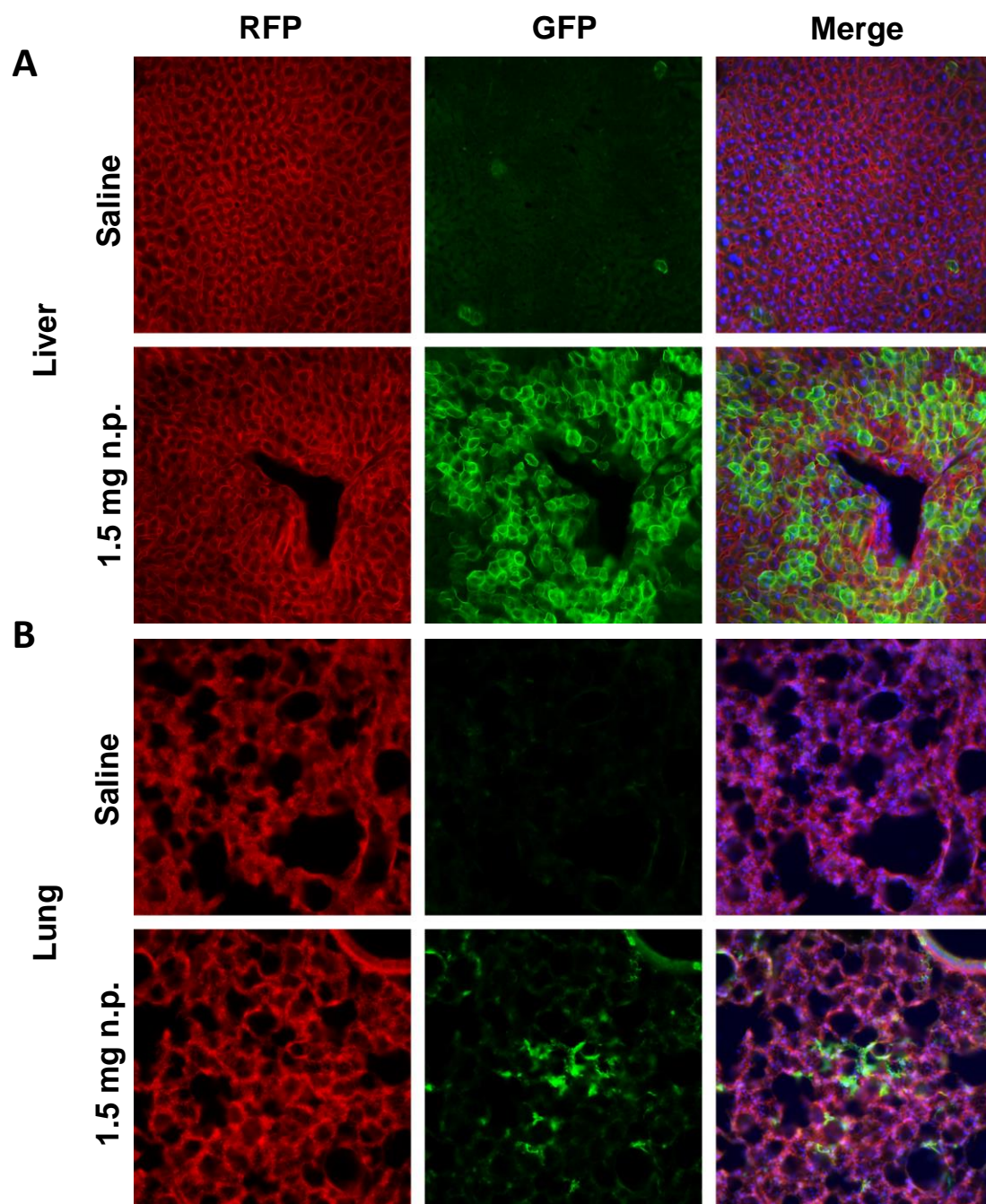
It is proposed that tamoxifen-loaded PLGA nanoparticles could be utilized to selectively activate tamoxifen-inducible Cre in the liver, thereby causing liver-specific excision of the mT/mG reporter construct. One potential route of administration was intravenous (tail vein) injection. Tamoxifen-loaded nanoparticles (1.5 mg nanoparticles per 25 g mouse) or vehicle (saline) were administered i.v., and fixed tissues were collected for frozen sectioning and imaging 7 days later, as described in Chapter II (Experimental Procedures). Compared to vehicle control, i.v. nanoparticle administration robustly induced expression of mG in the liver (Figure 5.4A), though not as robustly as i.p. tamoxifen administration (Figure 5.2). However, Cre-mediated excision was not liver-specific. For example, Figure 5.4B illustrates that Cre-mediated excision and subsequent mG expression was also seen in the lung compared to vehicle control.

4. Intrasplenic injection of tamoxifen-loaded PLGA nanoparticles selectively induces expression of mG in the liver.

Previous studies have reported that PLGA nanoparticles accumulate in the liver after intrasplenic injection, with a high percentage of injected nanoparticles accumulating in Kupffer cells (89). Therefore, tamoxifen-loaded

Figure 5.4: Native green fluorescence in liver and lung after i.v. administration of tamoxifen-loaded PLGA nanoparticles.

(A) Representative photomicrographs (200x) of native red (left) and green (middle) fluorescence in liver sections after i.v. administration of vehicle (saline) or 1.5 mg tamoxifen-loaded PLGA nanoparticles. (B) Representative photomicrographs (200x) of native red (left) and green (middle) fluorescence counterstained with DAPI in lung sections after i.v. administration of vehicle (saline) or 1.5 mg tamoxifen-loaded PLGA nanoparticles. Merged images are shown in the right column.



PLGA nanoparticles were injected at increasing concentrations (0-1.5 g/mouse) intrasplenically in R26CreER⁺/mTmG⁺ mice. Membrane-targeted eGFP was detected immunohistochemically in the liver and lung (Figure 5.5). Some cases of spontaneous mG expression was observed in animals receiving vehicle (saline) alone in both the liver and lung (Figure 5.5, top panels). In the liver, intrasplenic injection of nanoparticles produced a dose-dependent increase in GFP staining (Figure 5.5, right panels). GFP staining appeared in both hepatocytes and non-parenchymal cells. There was no observable effect of nanoparticle injection on GFP staining in the lung (Figure 5.5, left panels).

To quantify mG expression in various tissues, mRNA expression of *Egfp* was measured by qRT-PCR (Figure 5.6A). In lung, kidney, and spleen tissue homogenates, *Egfp* expression was unaffected by tamoxifen NP injection. Alternatively, in the liver, *Egfp* expression was significantly induced approximately 2 fold after 0.75 mg tamoxifen NPs, and induced ~10 fold at the highest dose of tamoxifen NP. To further confirm tamoxifen-mediated excision, native fluorescence of mT (red fluorescence) and mG (green fluorescence) was detected in frozen liver and lung sections (Figure 5.6B). In the lung, red fluorescence was observed ubiquitously with no detectable green fluorescence. In the liver, red fluorescence also predominated, however, clear green fluorescence was also observed throughout the tissue.

Figure 5.5: Membrane-targeted eGFP expression after intrasplenic injection of tamoxifen-loaded PLGA nanoparticles in liver and lung.

Representative photomicrographs (200x) of eGFP detected immunohistochemically after intrasplenic administration of tamoxifen-loaded PLGA nanoparticles (0.375, 0.75, or 1.5 mg per 25 g mouse) or vehicle (saline) in formalin-fixed, paraffin-embedded lung and liver sections

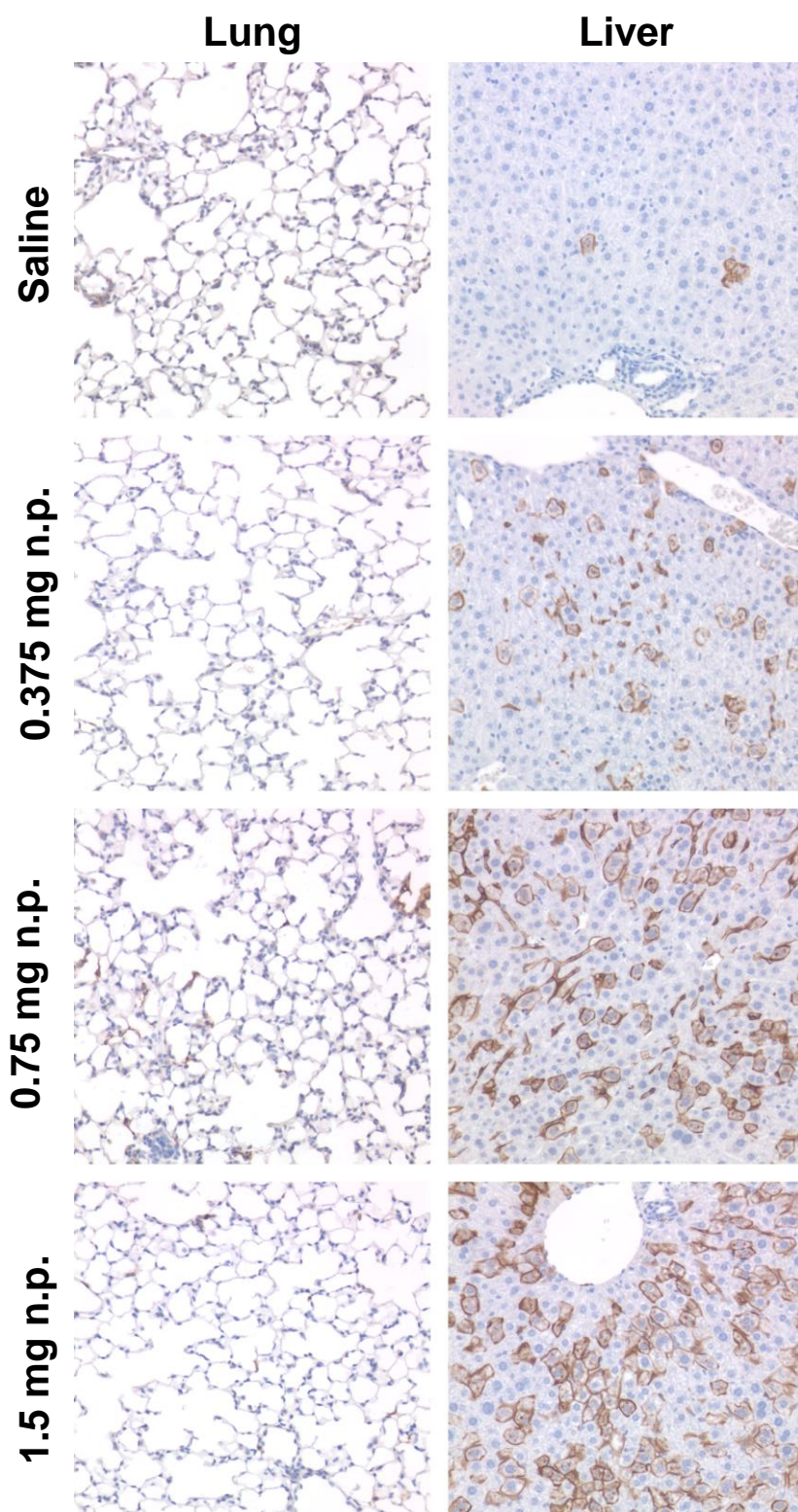
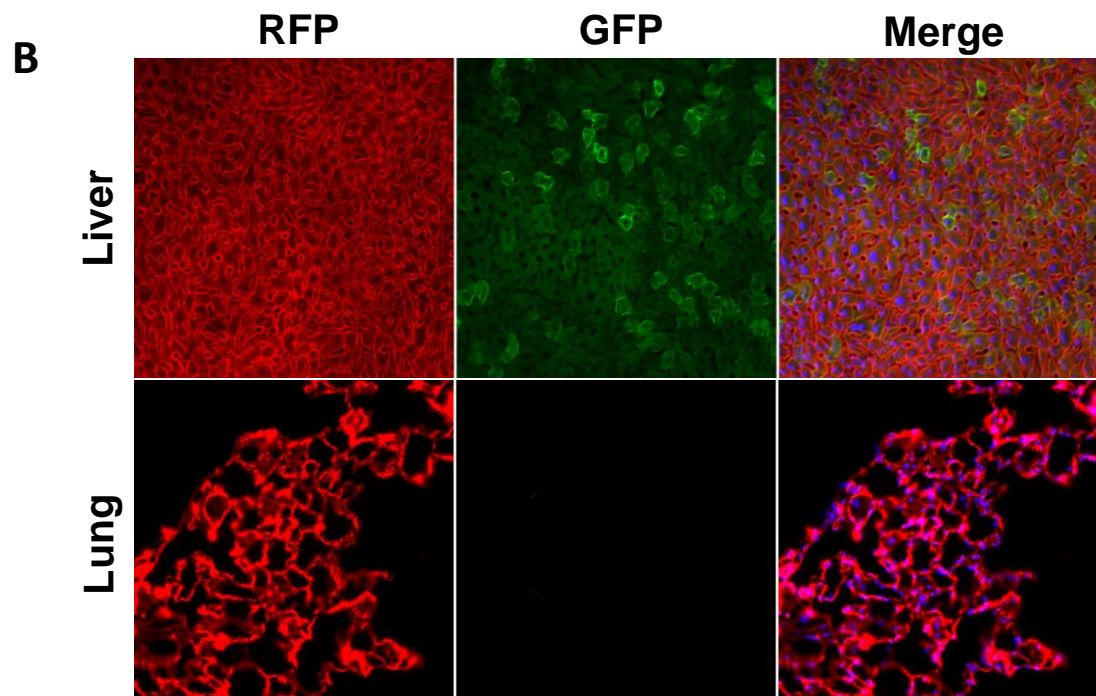
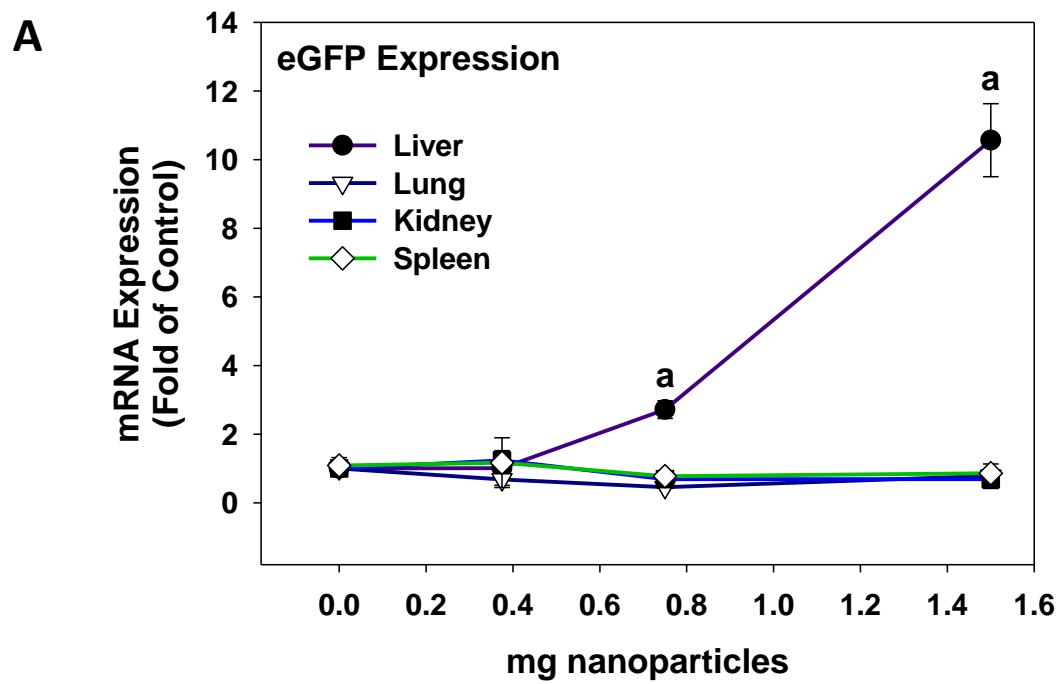


Figure 5.6: Enhanced-GFP expression in liver, lung, kidney, and spleen following intrasplenic administration of tamoxifen-loaded PLGA nanoparticles.

(A) mRNA expression of *Egfp* measured via qRT-PCR in liver, lung, kidney and spleen tissue homogenates of animals injected intrasplenically with 0, 0.375, 0.75, or 1.5 mg tamoxifen-loaded PLGA nanoparticles. (B) Representative photomicrographs (200x) of native red and green fluorescence in frozen liver and lung sections following intrasplenic administration of 0.75 mg tamoxifen-loaded PLGA nanoparticles.



D. Discussion

The goal of this study was to develop an in-vivo system to determine the role of hepatic-derived mediators, such as cytokines and chemokines, in propagating injury in other organs. Interactions between the liver and other organs have been well-characterized in certain disease models, e.g., the “gut-liver axis” in alcoholic liver disease. Current approaches used to target the liver are limited in specificity (the cell population they target) and the persistence of the effect. For example, liver-specific Cre-lox approaches, such as albumin-driven Cre, target only hepatocytes and are therefore insufficient to interrogate the role of Kupffer cells- the hepatic cell type which produces a majority of inflammatory mediators. Other methods to target the liver, such as viral vectors (e.g., rAd and rAAV), are limited by poor transduction efficacy (rAAV) or relatively brief transduction (rAd). Methods to selectively target Kupffer cells (versus other hepatic cells) include transgenic approaches, including LysM-Cre transgenics and ‘Kupffer cell chimeras’ have also been developed. Although these methods are relatively selective for hepatic macrophages versus other hepatic cell types, both tend to target extrahepatic macrophages. Therefore, the current study sought to develop a targeted transgenic approach to selectively modify the genetics of hepatic macrophages while avoiding macrophage populations in other tissues, such as the lung.

To achieve this goal, a double transgenic (i.e., Cre-LOX) approach was employed, wherein Cre induction was controlled by an external stimulus (R26CreER⁺/mTmG⁺). In this strain, systemic tamoxifen administration will

transduce nearly every cell in the organism (Figure 5.2). Restriction of the induction of Cre was facilitated by selective delivery of tamoxifen to the target cells with nanoparticles. The liver in general, and Kupffer cells in particular, are well known targets of injected nanoparticles. Indeed, several of the modifications to the current generation of nanoparticles are designed to avoid the RES and the liver (145). Thus, nanoparticles employed in this study were 'reverse engineered' to accomplish exactly the opposite goal.

Based on this previously published work, it was expected that i.v. injection of nanoparticles would accumulate in the liver and thereby selectively transduce that organ. It was therefore initially somewhat surprising that i.v. injection of nanoparticles, although effective at transducing the liver, also had off-target effects in other organs (e.g., lungs; Figure 5.4). These data may not be mutually exclusive with previous findings; the lungs are the first capillary bed that an intravenous agent contacts. Furthermore, final accumulation of nanoparticles determined in previous work may differ from sites wherein the particles accumulate at least transiently. Nevertheless, a more liver-specific delivery method was developed employing intrasplenic injections. Previous work has shown that PLGA nanoparticles, when injected intrasplenically, accumulate in Kupffer cells (89). Indeed, intrasplenic injection of tamoxifen-loaded PLGA nanoparticles induces dose-dependent expression of *Egfp* in the liver, but did not induce expression in the lung, kidney, or spleen (Figures 5.5 and 5.6). Interestingly, both hepatocytes and Kupffer cells were transduced.

The implications of these results are far-reaching. First, these experiments have developed a tool to discern the specific role of liver-derived cytokines in injury in other organs. Although hepatocyte-specific promoters exist, many cytokines are derived from the resident hepatic macrophages, the Kupffer cells. To date, no Kupffer cell-specific promotor has been identified that selects for this cell type over macrophages in other tissues. Furthermore, liver-derived products may be sourced from several hepatic cell types. For example, the release of TNF α into the systemic circulation is controlled by TNF α converting enzyme (TACE), which is derived from both hepatocytes and Kupffer cells. To study the role of hepatic TNF α on extrahepatic injury, a flox-flanked TACE (TACE^{flox}) mouse could be employed. Selective deletion of this enzyme could, in theory, prevent the release of TNF α from the liver into systemic circulation while not affecting local, paracrine effects of TNF α in the liver. Systemic TNF α is proposed to contribute to injury in the lung (35) and brain (146), among others, and cytokines that may be of hepatic origin have been implicated in multiple organ failure in a variety of disease states, including decompensated liver disease, systemic inflammatory response syndrome (SIRS) (82), and acute pancreatitis (147). The system described here would be useful for determining the specific origin of cytokines of interest. Finally, the development of this tool has implications beyond the scope of basic research. Clinically, identifying the role of hepatic-derived cytokines in disease in other organs provides novel insight for the treatment of these conditions. For example, these data provide support for

identifying underlying liver disease as a screening and/or treatment goal in patients at risk for developing multiple organ failure.

This approach, however, is not without limitation. First, this approach requires intrasplenic administration of nanoparticles, and therefore requires moderate technical skill to perform the necessary surgical procedure. Next, although it was histologically observed that Cre-mediated mG expression was robustly induced in Kupffer cells, mG expression was also noted in hepatocytes and possibly other non-parenchymal cells. The current approach will therefore not be sufficient to specifically target Kupffer cells while avoiding other hepatic cell types. This limitation, however, may actually be advantageous in some scenarios. Additionally, this approach could be more selective by employing a Kupffer-cell specific promotor to drive expression of Cre. Indeed, the C-type lectin CLEC4F has been characterized as a Kupffer cell-specific gene (148). However, no commercially available Cre-expressing mouse driven by this promotor is currently available. Third, this model does not address the possibility that communication between the liver and other organs may be bi-directional. Indeed, in experimental studies of ventilator-induced ALI, data suggests mediators released from the lung may damage tissues in other organs, including the liver (149, 150). However, the dose and route of administration of nanoparticles could be easily altered to target macrophage populations in other tissues. For example, direct administration of nanoparticles to the lung (e.g., intratracheal instillation or oropharyngeal aspiration) would likely selectively target alveolar macrophages, while avoiding hepatic macrophages. Finally, the

current system will only address the role of inflammatory mediators released from the cells that are residing in the liver at the time of intrasplenic nanoparticle injection. As such, inflammatory cells recruited to the liver at any point in the disease model after nanoparticle administration will likely be unaffected. This limitation, however, may also be a strength because it allows for separation of the effects of resident versus recruited macrophages on systemic release of inflammatory mediators.

In conclusion, the findings presented here describe a novel, in-vivo approach to selectively edit the genetics of hepatic cells, including hepatocytes and Kupffer cells. Using animals expressing tamoxifen-inducible Cre recombinase, any flox-flanked gene of interest may be excised following targeted delivery of the inducing agent. This system has potential applications for research in any disease state in which hepatic-derived cytokines or other signaling molecules are implicated, such as alcohol-enhanced ALI or shock-induced multiple organ failure. Such studies may provide new insight for the role of underlying liver injury or inflammation in the diagnosis and treatment of disease in other organs.

CHAPTER VI

DISCUSSION AND CONCLUSIONS

A. Restatement of goals and questions

The overall goal of the work described in this dissertation was to expand on the unifying hypothesis that not only do the liver and lung share parallel mechanisms of injury in response to alcohol exposure, but that injury in these two systems may be interdependent. The Arteel group has demonstrated that PAI-1 and fibrin ECM deposition are critical in alcohol-induced liver injury. The experiments in Chapter III were performed to determine the role of this mechanism of injury in alcohol-enhanced endotoxemia-induced ALI, thereby identifying potential parallel mechanisms of injury in the liver and lung. Chapter IV characterized lung injury and inflammation in a model of acute-on-chronic alcohol exposure originally developed to study alcoholic liver disease, thereby laying the groundwork for further exploring parallel mechanisms of injury. Finally, Chapter V describes the development of an inducible transgenic animal model to selectively alter the genetics of hepatic macrophages to test the hypothesis that hepatic-derived mediators may contribute to lung injury. Taken together, these studies provide new insight into the complex mechanisms of alcohol-related

acute lung injury by building on well-described mechanisms of liver injury, and also develop a novel transgenic animal model to explore the communication between these two organs.

B. Major findings of this dissertation

1. PAI-1-mediated fibrin accumulation and platelet aggregation play a critical role in alcohol-enhanced experimental ALI.

Previous studies from the Arteel group have demonstrated that PAI-1 plays a critical role in alcohol-induced liver injury. Genetic deletion of PAI-1 protected against alcohol-induced inflammation and necrosis in a model of chronic enteral ethanol administration in mice, as well as in LPS-induced liver injury after acute ethanol exposure. Additionally, PAI-1 has been shown to be involved in the development of LPS-induced acute lung injury, as well as other forms of lung injury, such as bleomycin-induced pulmonary fibrosis. However, the role of PAI-1 in alcohol-enhanced ALI has not been previously determined. The goals of Chapter III were three-fold: 1) to establish if PAI-1 and its downstream targets are up-regulated in alcohol-enhanced acute lung injury, 2) to determine the role of PAI-1 in injury and inflammation in this disease state and 3) to establish a potential mechanism by which PAI-1 and its downstream targets contribute to alcohol-enhanced ALI.

First, to investigate this potential parallel mechanism of liver and lung injury, PAI-1 expression and fibrin accumulation were measured in the lung tissue of animals receiving LPS injection with chronic ethanol pre-exposure (a previously established model of alcohol-enhanced ALI) (35). The studies in

Chapter III determined that, in fact, chronic ethanol pre-exposure exacerbated *Pai-1* mRNA induction and protein expression after LPS administration. As discussed in detail in Chapter I Section A.5, induction of PAI-1 impairs fibrin degradation by inhibiting plasmin. The Arteel group has previously demonstrated that blocking fibrin accumulation in the liver by knocking out PAI-1 or inhibiting thrombin cleavage protected against alcohol-enhanced inflammation (38). Therefore, fibrin accumulation in the lung was also assessed. Similar to the phenomena observed in the liver, chronic ethanol pre-exposure exacerbated fibrin accumulation in the lung tissue.

Fibrin can accumulate in a tissue as a result of enhanced activation of the coagulation cascade (thrombin cleavage) or by impaired fibrin degradation (PAI-1 induction). In addition to the enhanced PAI-1 induction seen in the lung tissue in Chapter III, ethanol pre-exposure was also associated with enhanced thrombin activation. These data suggest that in this experimental setting, fibrin accumulation in the lung is promoted on “both sides” of the coagulation cascade, i.e., by both enhanced generation and impaired degradation. Fibrin matrices can be pro-inflammatory by a number of mechanisms, as discussed in Chapter I, Section A.5. One such mechanism is by acting as a substrate for the adhesion of inflammatory cells, including platelets. In Chapter III, LPS administration, both alone and with alcohol pre-exposure, caused accumulation of platelets in the lung. Next, the causal role of PAI-1 induction on fibrin deposits and platelet accumulation was determined using PAI-1-deficient mice. As expected, PAI-1

deletion attenuated pulmonary fibrin deposition. Interestingly, the loss of fibrin was associated with complete mitigation of platelet accumulation.

The second goal of this study was to determine the role of PAI-1 and its downstream targets in injury and inflammation in alcohol-enhanced ALI. To this end, lung injury and inflammation were assessed in ethanol and/or LPS-treated wild-type or PAI-1 knockout mice. As expected, ethanol pre-exposure exacerbated endotoxemia-induced ALI in wild-type mice. Interestingly, in a finding similar to that of previous studies in the liver, PAI-1-deficient animals were protected against alcohol-enhanced edematous lung injury. Alcohol-enhanced alveolar septal thickening and BAL total protein levels were completely attenuated in mice lacking PAI-1. Interestingly, pulmonary neutrophil accumulation was unaffected by ethanol pre-exposure or PAI-1 deficiency, despite the effects of these variables on the measurements of injury assessed in this model.

Finally, the experiments in Chapter III were performed to suggest a proposed mechanism by which PAI-1 and its downstream targets are involved in alcohol-enhanced ALI. Even though the number of neutrophils was unaffected by diet or genotype, the activation state of these inflammatory cells may be altered by exposure to different cytokines and chemokines. Additionally, cytokines and chemokines themselves may also promote tissue damage (105). Therefore, mRNA expression of cytokines, chemokines, and vascular adhesion molecules was measured. PAI-1 deficiency attenuated the ethanol-enhanced expression of the chemokines MIP-2 (*Cxcl2*) and KC (*Cxcl1*), as well as the

vascular adhesion molecules *Icam-1* and *V-cam1*. Platelet-mediated signaling events can lead to expression of these chemokines upon aggregation and activation, and, interestingly, Chapter III illustrated that PAI-1 deficiency almost completely blocks LPS-induced platelet accumulation in the lung. Therefore, expression of MIP-2 and KC was measured in lung tissue of mice lacking integrin β_3 , the beta subunit of the platelet specific integrin $\alpha_{IIb}\beta_3$, a primary integrin receptor by which platelets adhere to fibrin matrices. Interestingly in β_3 knockout animals, ethanol-enhanced MIP-2 and KC expression was significantly blunted to levels similar to LPS administration alone, similar to what was seen in PAI-1-deficient animals. Interestingly, ethanol-enhanced PAI-1 expression was also attenuated in these β_3 knockout animals. Taken together, these results indicate that the mechanism whereby alcohol exaggerates LPS-induced lung injury requires PAI-1-mediated pulmonary fibrin accumulation, and suggest a novel mechanism whereby alcohol contributes to inflammatory ALI by enhancing fibrinogen-platelet engagement.

Text Box 6.1

Take-home points

- *Alcohol pre-exposure exacerbates PAI-1 induction and fibrin deposition in the lung. LPS injection causes pulmonary platelet accumulation.*
- *Knocking out PAI-1 almost completely attenuated fibrin and platelet accumulation. Furthermore, these PAI-1-deficient mice were protected against alcohol-enhanced ALI.*
- *Blocking PAI-1-mediated fibrin accumulation, as well as platelet adherence to fibrin (β_3 knockout animals), mitigated chemokine expression in the lung, suggesting a possible novel mechanism of alcohol-enhanced ALI.*

2. Chronic + Binge alcohol exposure promotes inflammation in the lung and alters pulmonary function.

As described in the Chapter I Section 6, a major limitation of in vivo studies investigating the toxic effects of alcohol on the lung is the mouse models which are often employed. Indeed, ad libitum ethanol exposure models, including ethanol in drinking water and chronic Lieber-DeCarli liquid diet produce relatively low blood alcohol levels and mild, if any, clinically relevant liver pathology. Recently, a new model of acute-on-chronic alcohol exposure was developed to study alcohol-induced liver disease (80), in which chronic dietary exposure is followed by an acute bolus gavage of alcohol. It is hypothesized that this model better recapitulates the drinking patterns of an individual with an alcohol use disorder. Importantly, hepatic pathology in this acute-on-chronic model is more similar to human alcoholic liver disease. The experiments described in Chapter IV characterize the previously uninvestigated effects of this alcohol exposure regimen in the lung. As such, the goals of the experiments in Chapter IV were to 1) characterize lung injury and/or inflammation in this new, perhaps more relevant model of alcohol exposure, 2) to determine what, if any, functional effects this ethanol exposure pattern has on pulmonary function, thereby 3) determining if alcohol exposure alone, absent any secondary injury, is sufficient to cause pulmonary inflammation or injury.

Liver injury and inflammation in the acute-on-chronic, or chronic + binge, has been previously well-characterized as resembling alcoholic steatohepatitis, with fat accumulation, inflammation, and necrosis. These findings were

recapitulated in Chapter IV. While mice administered 10 days of ethanol-containing liquid diet alone showed little liver pathology, as seen histologically or biochemically, 10 days of alcohol feeding followed by ethanol binge (chronic + binge ethanol) dramatically exacerbated liver injury and inflammation. As previously characterized, this inflammation was predominately neutrophilic in nature, more closely resembling the human pathology. The effects of chronic + binge ethanol exposure have been uncharacterized in the lung, and the first goal of this study was to address this question. Similar to what is seen in the liver, 10 days of ethanol-containing liquid diet produced no overt pathologic effects in the lung, i.e., no inflammatory cell recruitment or alterations in tissue architecture. Alternatively, chronic + binge ethanol exposure promoted recruitment of neutrophils, which were seen sequestered in the lung parenchyma 9 hours after ethanol binge. This neutrophil recruitment was associated with significant induction of the neutrophil chemokines *Cxcl1*, *Cxcl2*, and *Cxcl15*. This inflammation was persistent. Indeed, although 24 hours after ethanol binge, the neutrophil infiltration in the lung tissue had decreased (data not shown), the number of cells recovered in the BALF was significantly elevated. This increase in total BAL cells was driven primarily by an increase in neutrophils. Indeed, the total number of BAL neutrophils was significantly elevated in mice exposed to chronic + binge ethanol. The total number of lymphocytes was also significantly elevated in this group, although to a lesser extent.

Population studies have indicated that alcohol consumption may trigger asthma attacks in some individuals (135). However, data from experimental in-

vivo studies are unclear, and at times conflicting. Therefore, in accordance with the second goal of this study, the effect of chronic + binge alcohol exposure on pulmonary mechanics were measured. At baseline, ethanol exposure did not affect pulmonary mechanics. However, alcohol exposure caused airway hyperreactivity in response to inhaled methacholine, as demonstrated by increased airway resistance, Newtonian resistance, and respiratory resistance (the real part of respiratory impedance). These data suggest that acute-on-chronic alcohol exposure promotes hyperreactivity, likely in both the central airways and lung parenchyma. Taken together, these data address the third goal of this study- to determine if ethanol alone is sufficient to cause inflammation in the lung. The results of this study suggest that chronic + binge ethanol alone causes pulmonary inflammation and affects pulmonary mechanics.

Text Box 6.2

Take-home points

- *Chronic + binge alcohol exposure causes inflammation in the lung, marked by neutrophil infiltration in the lung tissue and BALF*
- *Airway resistance in response to inhaled methacholine is increased in animals exposed to chronic + binge alcohol*
- *Taken together, these data suggest that, based on dose and pattern of exposure, alcohol alone is sufficient to cause inflammation in the lung and alter pulmonary mechanics.*

3. Intrasplenic administration of tamoxifen-loaded PLGA nanoparticles selectively induces cre-mediated recombination in the liver.

Multiple organ failure is highly recognized as a hallmark of end-stage alcoholic liver disease. However, the role of organ-organ crosstalk in the early stages of disease is increasingly appreciated. In fact, studies have suggested that extra-thoracic cytokines, like TNF α , may contribute to alcohol-enhanced ALI. However, no experimental system is currently available to test this hypothesis. The goals of Chapter V of this dissertation were therefore 1) to identify a drug delivery method which selectively targets the liver versus the lung and 2) to develop a transgenic animal to demonstrate the feasibility of selectively inducing a transgene in the liver versus the lung.

To address the first goal, this study used tamoxifen-loaded PLGA nanoparticles and a dual-fluorescence reporter mouse to attempt to develop this tool. The mT/mG reporter construct expresses red fluorescent, membrane-targeted TdTomato (mT) before Cre-mediated excision. Upon induction of Cre, flox-flanked mT is deleted and membrane-targeted enhanced GFP (mG) is expressed. These animals were crossed with tamoxifen-inducible Cre (R26CreER) that is expressed in every cell. Therefore, any cell exposed to tamoxifen would express Cre, thereby inducing mT excision and mG expression. For example, when tamoxifen is injected intraperitoneally, every cell in the body expresses mG, as demonstrated in Chapter V. It was proposed that i.v. injection of PLGA nanoparticles would be sufficient to target hepatic macrophages. While mG was robustly induced in the liver upon examination of green fluorescence in

frozen tissues, it was observed that mG was also induced in the lung, indicating that tamoxifen-loaded PLGA nanoparticles must have also targeted the lung.

After i.v. (tail vein) administration of a pharmacologic agent, the first capillary bed that agent contacts is the lung. This property may explain why green fluorescence was observed in both the liver and lung. As such, a more liver-specific delivery method was required. Blood from the spleen empties directly into the hepatic portal vein, thereby making intrasplenic injection an, essentially, direct route of administration of pharmacologic agents to the liver. Furthermore, the majority of PLGA nanoparticles, when injected intrasplenically, have been shown to accumulate in Kupffer cells (89). It was therefore proposed that intrasplenic administration of PLGA nanoparticles would induce Cre-mediated excision in the liver, while avoiding off-target organs like the lung.

As such, to address the second goal of this study, tamoxifen-loaded PLGA nanoparticles were injected intrasplenically in R26CreER⁺/mTmG⁺ mice. Interestingly, the experiments in Chapter V demonstrate that this method induces dose-dependent expression of *Egfp* in the liver, but did not induce expression in the lung, kidney, or spleen when compared to injection of vehicle control. At all doses of nanoparticles injected, both hepatocytes and non-parenchymal cells appeared to express mG. Taken together, the data from Chapter V indicate that liver-specific (intrasplenic) delivery of tamoxifen-loaded PLGA nanoparticles may be sufficient to selectively induce Cre-mediated excision in hepatic macrophages while avoiding macrophage populations in other tissues, including the lung.

Take-home points

- *Chapter V identified a drug delivery vehicle (tamoxifen encapsulated in PLGA nanoparticles) and a suitable reporter system (a dual-fluorescence reporter construct regulated by tamoxifen-inducible Cre to develop an in-vivo model to investigate the liver-lung axis.*
- *Intrasplenic, but not intravenous, administration of nanoparticles selectively induced Cre-mediated excision of the reporter construct in the liver, and not in the lung.*

C. Significance of new findings

As discussed in Chapter I, alcohol abuse is the number one risk factor for developing ARDS in the setting of sepsis. However, the mechanisms by which alcohol sensitizes the lung to development of ALI and ARDS are poorly understood. One of the primary aims of this dissertation was to utilize knowledge of established mechanisms in alcohol-induced liver injury to unmask novel mechanisms of alcohol-enhanced lung injury. The experiments in Chapter III identify a novel role for the fibrinolytic inhibitor, PAI-1, and by extension, fibrin accumulation and integrin β_3 - mediated platelet aggregation. The results from this study suggest that alcohol pre-exposure enhances PAI-1 induction after LPS administration, thereby promoting accumulation of fibrin in the lung tissue, which acts as the ligand for platelet aggregation via platelet integrin $\alpha_{IIb}\beta_3$. This study revealed several potential druggable targets which could be explored for prevention of the development of ARDS in at-risk individuals. For example, anti-

coagulants are already being actively clinically explored as treatment options for sepsis-induced ARDS (151). Additionally, platelet aggregation inhibitors are FDA approved and readily clinically available. The studies presented in Chapter III, therefore, are just one example of how understanding parallel mechanisms of liver and lung injury may provide new treatment options for at-risk patients.

Murine models are useful tools to investigate mechanisms by which alcohol pre-exposure promotes injury and inflammation in target organs, including the liver and the lung. However, many animal models employed are limited in their ability to recapitulate risky human drinking patterns and the human pathologies that are associated with them. The recently developed “acute-on-chronic” or “chronic + binge” mouse model of alcohol exposure more accurately reproduces risky human drinking behavior, in which episodes of binge drinking are coupled with alcohol abuse. Liver pathology in this model of alcohol exposure is well characterized (152). This pattern of ethanol exposure in mice produces a phenotype more similar to human alcoholic steatohepatitis, including hepatic fat accumulation, neutrophil infiltration, and necrosis. Novel findings in Chapter IV show that chronic + binge alcohol exposure also promotes inflammation and airway hyper-responsiveness in the lung. This work has characterized the effects of a new model of ethanol exposure in mice, unmasking lung pathologies that are not seen with different models of ethanol administration. Importantly, this study is the first to demonstrate that ethanol exposure alone, absent any exogenous inflammatory insult (e.g., LPS), is sufficient to induce inflammation in the lung. Additionally, population studies have indicated that in some individuals, alcohol

consumption can trigger asthma attacks. However, these episodes have traditionally been linked to impurities in alcoholic beverages and/or acetaldehyde detoxification insufficiencies. The experiments in Chapter IV indicate that ethanol alone may contribute to mild airway hyperresponsiveness. Taken together, these results indicate that this model may be used in the future to investigate how ethanol exposure may drive the development of a pro-inflammatory milieu in the lung and airways, thereby promoting injury. Once again, these studies represent another example of how understanding alcohol-induced toxicity in one organ, such as the liver, may provide novel insight into a second target organ, like the lung.

Finally, the third Aim of this dissertation sought to develop a tool that could be used to empirically assess the role of hepatic, or more specifically, Kupffer-cell derived cytokines in the development of alcohol-enhanced acute lung injury. The experiments in Chapter V demonstrate that when tamoxifen-loaded PLGA nanoparticles are delivered directly to the liver of animals expressing tamoxifen-inducible Cre recombinase, Cre-mediated excision occurs exclusively in the liver, with recombination occurring in both hepatocytes and non-parenchymal cells, including Kupffer cells. These results are significant for several reasons. First, these experiments have developed a tool to discern the role of hepatic-derived cytokines in alcohol-enhanced acute lung injury. To date, no Kupffer cell-specific promotor has been identified that exclusively targets resident hepatic macrophages while avoiding macrophages in other tissues, including the lung. In the system developed in Chapter V, the role of any gene that can be flox-flanked

may be assessed. For example, to determine the role of hepatic-derived TNF α in alcohol-enhanced ALI, the commercially-available, flox-flanked TNF α converting enzyme (TACE^{flox}) mouse could be employed. Selective deletion of this enzyme could, in theory, prevent the release of TNF α from the liver into systemic circulation while not affecting local, paracrine effects of TNF α in the liver. Second, while communication between the liver and lung may be of most interest for the current study, the role of hepatic-derived cytokines in alcohol-induced toxicity is of great interest in other organs as well. For example, systemic TNF α is established to play a role in alcohol-induced brain inflammation (153). The system described in Chapter V would be useful for determining the origin of this systemic cytokine. Third, the applications of this system are not limited to the context of alcohol-induced organ toxicity. For example, hepatic macrophages have been widely implicated in multiple organ failure in acute pancreatitis (147). The system described in Chapter V could be utilized to investigate the specific role of any flox-flanked gene in hepatic macrophages in this disease. Finally, the development of this tool has implications beyond the scope of basic research. Clinically, identifying the role of hepatic-derived cytokines in alcohol-induced toxicity in the lung, as well as other organs, provides novel insight for the treatment of these conditions. For example, these data provide support for identifying underlying liver disease as a screening and/or treatment goal in patients at risk for developing ARDS, as well as other systemic diseases.

D. Strengths and weaknesses

1. Strengths

There are several strengths of the work presented in this dissertation. First, this work uses relevant whole animal models to address the Aims presented here. The effects of alcohol use are complex phenomena which are not limited to a single organ or cell type, directly supporting the necessity of the work presented in this dissertation which investigates parallel mechanisms of injury in the liver and lung, as well as the potential interdependence of these two organs, during alcohol exposure. The questions explored here could likely not be sufficiently addressed by the use of other model systems (e.g., cultured cells). In particular, this study explores the effects of a new, relevant mouse model of ethanol exposure in the lung. Chronic + binge ethanol exposure has been characterized as, perhaps, a more clinically-relevant mouse model of ethanol exposure. Blood alcohol levels achieved in this model, as well as the liver pathology it produces, are similar to that of individuals at risk of developing alcohol-induced disease. For example, increased risk of ARDS only occurs in patients consuming more than three drinks per day (127). For the first time, the work in this dissertation explores the effects of chronic + binge alcohol exposure on the lung, and produced the novel finding that, under these conditions, ethanol alone is sufficient to cause pulmonary inflammation and alter lung mechanics.

In addition to exploring alternative alcohol exposure models to investigate the effects of ethanol on the lung, this dissertation also developed a new tool to

gain novel insight in the liver-lung axis of disease. The wide range of applications for this new tool is an obvious strength of the work presented in this dissertation. The use of a tamoxifen-inducible ubiquitous promotor allows for targeted excision of any gene of interest that is flox-flanked. As mentioned previously, targeted deletion of TNF α converting enzyme (TACE) from the liver, specifically hepatic macrophages, would prevent the release of TNF α from the liver into systemic circulation and, if the hypothesis is correct, protect against alcohol-enhanced acute lung injury. However, even if this hypothesis is insufficient, the system developed here would allow for selective deletion of other genes, and depending on the nanoparticle delivery method, selective deletion of genes from other macrophage populations.

Finally, this dissertation has identified a new mechanism of alcohol-enhanced ALI. Building on knowledge of the role of the protease inhibitor, PAI-1 in alcohol-induced liver injury, this dissertation sought to explore the idea that similar mechanisms involving transitional ECM remodeling may be involved in alcohol-enhanced lung injury. Using an established model of experimental endotoxemia with ethanol pre-exposure, the experiments in Chapter III demonstrated a novel role for PAI-1 and subsequent fibrin accumulation and platelet aggregation. While platelets have been implicated in other forms of lung injury (103), the involvement of platelets in alcohol-enhanced ALI has not been explored. This dissertation has identified novel, druggable targets to prevent the progression of ARDS, such as PAI-1, fibrin, and platelet aggregation (potentially via integrin $\alpha_{IIb}\beta_3$). In fact, treatment options targeting dysregulation of the

coagulation cascade (e.g., nebulized heparin) are already being investigated as promising treatment options in patients with ARDS (154)

2. Weaknesses

Although the animal models employed in this dissertation are a key strength of this body of work, they also add a source of complexity. First, because a conditional (i.e., flox-flanked) PAI-1 knockout mouse is not currently commercially available, whole-body knockouts were used. This approach, unfortunately, does not allow for the assessment of the effect of the deletion in a tissue of interest, in this case, lung, or a specific cell type. It is possible, therefore, that effects of PAI-1 deletion observed in this model may be due to the modulation of PAI-1's pro-inflammatory effects in other tissues, such as the liver.

In addition to specific limitations of the animal models used, there is always an element of uncertainty when using animal models to attempt to recapitulate human disease. Although in-vivo research is certainly preferred to simpler models, such as cultured cells, to both the effects of chronic alcohol exposure on a specific tissue (the lung) as well as organ-organ crosstalk during chronic alcohol exposure, no animal model can completely recapitulate the human condition. Indeed, there is currently no rodent model that exactly represents the pattern of progression of alcoholic liver disease or alcohol-enhanced susceptibility to ALI, or all of the clinical sequela associated with these disease states. Although increased PAI-1 levels has been associated with increased mortality in ARDS patients (52), there are currently no clinical studies

investigating if ARDS patients with a history of alcohol abuse have elevated plasma or BALF levels of PAI-1 compared to patients who do not have an alcohol use disorder. Additionally, the “chronic + binge” mouse model of alcohol exposure employed in this dissertation more accurately reproduces characteristics of human alcoholic liver disease, including hepatic neutrophil infiltration and inflammation. However, the effects of this alcohol exposure pattern have not been examined clinically in the lung. Indeed, the pulmonary inflammation and functional effects presented in mice in this dissertation were relatively mild. As such, if this low-grade inflammation were to occur in the human population, it may remain clinically silent in routine examination (i.e., if no obvious lung injury has occurred). Therefore, a direct correlation of the findings in Chapter IV to a human population is difficult to determine.

This dissertation also has limitations in terms of the experimental approaches that were employed. For example, the experiments in Chapter III use genetic modification (PAI-1 knockout and Integrin β_3 knockout) to investigate the role of PAI-1, fibrin accumulation, and platelet aggregation in alcohol-enhanced ALI. Based on the results of this study, it was concluded that ethanol-enhanced PAI-1 induction in the lung may contribute to exacerbated fibrin deposition and platelet aggregation, and these aggregated platelets may contribute to lung injury by driving chemokine expression and/or inflammatory damage in the tissue. However, this study did not directly test the role of platelets themselves. For example, platelet depletion using a depleting antibody, or administration of platelet activation inhibitors, such as clopidogrel, would more directly address the

role of platelets in this proposed mechanistic pathway. Furthermore, the approach utilized in Chapter V relied on tamoxifen-inducible Cre-recombinase driven by a ubiquitous promotor paired with liver-specific delivery of tamoxifen to selectively modify the genetics of Kupffer cells. However, although it was histologically observed that Cre-mediated mG expression was robustly induced in Kupffer cells, mG expression was also noted in hepatocytes and possibly other non-parenchymal cells. The current approach outlined in Chapter V will therefore, likely not be sufficient to specifically target Kupffer cells while avoiding other hepatic cell types.

Finally, the primary goal of the experiments in Chapter V was to develop an animal model that may be used to interrogate the role of hepatic-derived cytokines in alcohol-enhanced ALI. This model, therefore, does not address the possibility that communication between the liver and lung may be bi-directional. Indeed, in experimental studies of ventilator-induced ALI, data suggests mediators released from the lung may damage tissues in other organs, including the liver (149, 150). One advantage of the system developed in Chapter V is that the dose and route of administration of nanoparticles could be altered to target macrophage populations in other tissues. For example, direct administration of nanoparticles to the lung (e.g., intratracheal instillation or oropharyngeal aspiration) would likely selectively target alveolar macrophages, while avoiding hepatic macrophages. Additionally, the current system will only address the role of inflammatory mediators released from the cells that are residing in the liver at the time of intrasplenic nanoparticle injection. Recent studies have suggested

chronic alcohol administration causes infiltration of monocyte-derived macrophages into the liver (155). While the exact role of these infiltrating inflammatory cells, compared to that of the macrophages residing in the liver, is still widely debated, it is proposed that at least a subpopulation of the recruited macrophages promote inflammatory tissue damage (156). Although the system proposed in Chapter V may be limited by its inability to target macrophages that are recruited after the time of injection, this limitation may also be a strength because it allows for separation of the effects of resident versus recruited macrophages on systemic release of inflammatory mediators. However, it is possible that blocking release of inflammatory mediators into circulation may attenuate the recruitment of inflammatory cells into the liver. Future studies should consider the limitations presented here to further investigate these important research questions.

Summary Table 6.1

Strengths	Limitations
<ul style="list-style-type: none"> • Relevant animal model of chronic +binge alcohol exposure unmasks alcohol-induced pathology in lung • Novel approach to investigate liver-lung interactions has a wide range of applications, inside and outside the context of this dissertation • Clinically relevant, new, druggable targets in alcohol-enhanced ALI for at-risk patients identified. 	<ul style="list-style-type: none"> • Pathologies identified in animal models used here remain uninvestigated in human patients. • Liver-specific, transgenic system targets multiple hepatic cell types and does not address potential for bi-directional communication • Exact role of platelets in this novel mechanism of ALI requires further investigation.

E. Future Directions

While the experiments described in this dissertation answered specific gaps in the field's knowledge of the alcoholic lung phenotype and the liver-lung axis of disease, it has also created new questions to be addressed in future studies. Some of these questions are addressed below.

1. What is the role of platelet activation in alcohol-enhanced ALI?

The experiments performed in Chapter III identified fibrin-mediated platelet aggregation as a mechanism by which alcohol enhances endotoxemia-induced ALI. Deletion of the β subunit of integrin $\alpha_{IIb}\beta_3$, an integrin involved in platelet adherence to fibrin, significantly blocked alcohol-enhanced chemokine expression and PAI-1 expression. A logical next step for future experiments would be to directly test the role of platelets and platelet activation in the development of the alcoholic lung phenotype. This question could be addressed by various approaches. First, to more broadly test the hypothesis that platelet accumulation promotes chemokine expression in the lung, platelets could be depleted from the whole animal by injection of an anti-CD41 monoclonal antibody (157). Next, platelet inhibitors could be used to more specifically interrogate various platelet functions. Integrin $\alpha_{IIb}\beta_3$ inhibitors, such as abciximab, prevents adhesion of platelets (158). Clopidogrel is an antagonist of the platelet ADP receptor, $P2Y_{12}$; administration of this drug could investigate the specific role of activated platelets. More "platelet-specific" genetic approaches could also be

utilized (159). For example, mice lacking protease-activated receptor-4 (PAR-4), the platelet receptor for thrombin, is also an available option to study platelet activation in alcohol-enhanced ALI. Indeed, thrombin, a potent activator of platelets, was elevated in the plasma of animals exposed to ethanol and LPS. It is proposed that blocking platelet activation would mitigate alcohol-enhanced cytokine and chemokine expression, and ultimately, inflammatory injury. Additionally, the effect of the pattern and dose of alcohol exposure (i.e., chronic alcohol versus chronic + binge alcohol) would be an interesting question to explore. Further investigating the contribution of platelets to alcohol-enhanced ALI would be an interesting future direction for this study.

Summary Table 6.2

Platelet activation in ALI?	
Rationale	Blocking platelet aggregation significantly attenuated alcohol-enhanced chemokine expression in the lung.
Approach	Platelets may be depleted entirely using monoclonal antibodies, or drugs and/or genetic modification can be used to target specific activation pathways.
Expected Results	Blocking platelet activation will mitigate alcohol-enhanced ALI.

2. To what extent is the mechanism identified in Chapter III involved in alcohol-induced liver injury?

A major goal of this dissertation was to utilize established mechanisms of alcohol-induced liver injury to explore new questions about alcohol-enhanced ALI. The Arteel group found that PAI-1 induction and subsequent fibrin accumulation were critical in the development of liver injury and inflammation in the setting of chronic alcohol exposure (47), as well as acute alcohol-enhanced

LPS-induced liver injury (38). It was therefore proposed that similar mechanisms may be involved in alcohol-enhanced ALI. During this investigation, a new mechanism of inflammation, potentially mediated by platelets, was uncovered. An interesting target for further investigation would be to determine if this is a parallel mechanism of injury in the liver. One study demonstrated that platelets accumulate in the periportal microcirculation after acute ethanol administration in mice (160). Paradoxically, thrombocytopenia, or low platelet levels, is associated with chronic liver diseases, including alcoholic cirrhosis. It is proposed by others, however, that thrombocytopenia in end-stage liver disease may be a product of decompensation and not necessarily involved in pathogenesis at this stage of disease (161). The involvement of platelets in the inflammatory phase of alcoholic liver disease is unknown, and would therefore be an interesting question for future study. To study this effect, the model of chronic alcohol exposure with experimental endotoxemia employed in this dissertation could be utilized, and platelet accumulation could be detected in the liver immunohistochemically, as described in the lung in Chapter III. If platelets are found to accumulate in the liver, similar approaches as described in the previous section could be employed to determine if platelet activation contributes to alcohol-induced liver injury. Based on the findings of this dissertation, it is expected that platelet aggregation may occur in the liver in this disease model, and that blocking this aggregation may attenuate alcohol-induced liver injury.

Summary Table 6.3

Role of platelets in alcohol-induced liver injury?	
Rationale	Blocking platelet aggregation significantly attenuated alcohol-enhanced chemokine expression in the lung.
Approach	Platelets may be depleted entirely using monoclonal antibodies, or drugs and/or genetic modification can be used to target specific activation pathways.
Expected Results	Blocking platelet activation may attenuate alcohol induced injury or inflammation.

3. What is the mechanism by which chronic + binge alcohol exposure causes airway hyperresponsiveness?

Population studies have indicated that alcohol consumption may trigger asthma attacks in certain individuals, including those with a genetic polymorphism that prevents them from efficiently detoxifying acetaldehyde (127). The effects of alcohol-induced damage, separate from alcohol intoxication itself, on lung mechanics has not been extensively characterized. The experiments outlined in Chapter IV demonstrate that chronic + binge alcohol exposure causes airway hyperresponsiveness, as indicated by exacerbated resistance, Newtonian resistance and respiratory resistance in response to inhaled methacholine. These measurements were performed 24 hours after ethanol binge, and animals had been fasted from ethanol. Therefore, it is unlikely that any effects on lung mechanics seen in this study were due to alcohol intoxication or toxic alcohol metabolites, such as acetaldehyde. Potential alternative mechanisms of airway hyperresponsiveness would, therefore, be of interest to explore in future studies.

Airway responsiveness to methacholine can be altered by a variety of factors. First, interruption of the bronchial epithelium may exacerbate airway resistance in response to methacholine by allowing the drug to more freely access the underlying smooth muscle (162). The bronchial epithelium is protected by layers of phospholipids, or surfactants. In one study, guinea pigs exposed to chronic ethanol had significantly reduced surfactant levels in BALF compared to control animals (163). Surfactant levels could be measured in animals exposed to chronic + binge ethanol. If found to be reduced, an intervention study could be performed to attempt to restore surfactant proteins using a number of experimental approaches, including administration of exogenous surfactant (164). If surfactant loss is involved in this phenomenon, restoring surfactant may mitigate alcohol-induced airway hyperresponsiveness.

A second potential mechanism of exacerbated airway resistance is the induction of oxidative stress (165). Indeed, oxidative stress is hypothesized to be a key player in the alcoholic lung phenotype (4). Indices of oxidative stress, such as glutathione depletion, could be measured in animals exposed to chronic + binge ethanol. If elevated, antioxidants, such as N-acetyl cysteine, could be administered to attempt to attenuate alcohol-induced airway hyperresponsiveness (166).

Pulmonary inflammation can also promote airway hyperresponsiveness (162). For example, the experiments in Chapter IV demonstrated that chronic + binge alcohol exposure promotes neutrophil recruitment to the lung tissue and airways. Neutrophils release reactive oxygen species, which may therefore be

one potential mechanism of airway hyperresponsiveness seen in this model, as previously discussed. Additionally, a study measuring cytokine and chemokine levels in BALF of patients with alcohol use disorders found that regulated on activation, normal T-cell expressed and secreted (RANTES/CCL5) levels positively correlated with the severity of the alcohol use disorder (167, 168). The role of this chemokine has been widely characterized in airway hyperresponsiveness in asthma and allergic airway inflammation (169). One potential future study could investigate levels of this chemokine in the BALF of animals exposed to chronic + binge alcohol. If found to be elevated, the role of this chemokine in alcohol-induced airway hyperresponsiveness could be investigated in a commercially-available knockout mouse.

Finally, there is evidence suggesting that dysfunction of the parasympathetic neurons innervating the airway may play a role in airway hyperresponsiveness, but the exact mechanism has not been characterized (170, 171). It is therefore possible that chronic + binge alcohol exposure may somehow alter the innervation of the airway, thereby promoting hyperresponsiveness. This hypothesis could be investigated via approaches that block innervation, such as vagotomy. Taken together, these mechanisms, including epithelial barrier dysfunction, oxidative stress, inflammation, and innervation, are all plausible targets for future investigation. Furthermore, it would be of interest to determine if other patterns of alcohol exposure, such as acute or chronic alcohol exposure, also promote airway hyperresponsiveness, or if this phenotype is unique to chronic + binge alcohol exposure.

Summary Table 6.4

Mechanism of airway hyperresponsiveness?	
Rationale	Chronic + binge ethanol caused increased airway resistance in response to inhaled methacholine. Several factors known to contribute to airway hyperresponsiveness, including surfactant loss, oxidative stress, inflammation, and innervation, have previously been associated with alcohol exposure.
Approach	Exogenous surfactant administration, antioxidant administration, genetic deletion of specific inflammatory cytokines, or vagotomy may be used to investigate these questions.
Expected Results	An intervention to target one of these mechanisms may block alcohol-induced airway hyperresponsiveness.

4. Does hepatic-derived TNF α contribute to the alcoholic lung phenotype?

The experiments described in Chapter V developed a new tool for targeted transduction of cells in the liver that avoided the lung. While the implications of this work are far-reaching, the most relevant to this dissertation is that this system could be used to determine the role of hepatic-derived cytokines in alcohol enhanced ALI. TNF α -converting enzyme (TACE) is an enzyme required for cleavage and systemic release of TNF α into the blood. Previous research from the Arteel group has suggested that extra-thoracic TNF α , at least in part, drives alcohol-enhanced inflammation in endotoxemia-induced ALI. The system developed in Chapter V could be easily used to selectively delete TACE from the liver in mice expressing tamoxifen-inducible Cre and flox-flanked TACE. The use of this system would target TACE deficiency to Kupffer cells and hyepatocytes and, if the overall hypothesis is correct, block TNF α signaling in the

lung. In fact, these are currently being generated, and preliminary studies are being performed.

Summary Table 6.5

Role of hepatic TNFα in alcohol-enhanced ALI?	
Rationale	Previous studies indicate extra-thoracic TNF α may contribute to alcohol-enhanced ALI.
Approach	Using the liver-specific transduction method developed in Chapter V, TACE could be selectively deleted from hepatocytes and Kupffer cells.
Expected Results	Liver-specific deletion of TACE would prevent TNF α from being released into circulation from the liver, preventing TNF α -responsive signaling in the lung.

F. Summary and conclusions

The overall goal of the work described here builds on the overarching hypothesis that the liver and lung share parallel mechanisms of alcohol-induced toxicity, and that liver injury may contribute, at least in part, to the development of the “alcoholic lung phenotype.” The experiments in Chapter III describe a new potential mechanism of alcohol-enhanced ALI driven by exacerbated fibrin accumulation and platelet aggregation, identifying platelets as a target for investigation of future study in both the liver and lung. Chapter IV characterizes the effects of a perhaps more relevant ethanol exposure pattern in the lung, building on findings that acute (binge) alcohol administration following chronic alcohol exposure causes exacerbated liver injury and inflammation. Future studies will seek to identify the mechanism by which chronic + binge alcohol causes airway hyperresponsiveness. Finally, Chapter V describes the development of a novel tool using liver-targeted delivery of tamoxifen-loaded nanoparticles to selectively delete a gene of interest from hepatic macrophages

that does not also target the lung. The implications for this system are far-reaching, and importantly, can be used to selectively delete inflammatory factors (e.g., TACE) from the liver to determine if mediators (TNF α) of hepatic origin drive injury in the lung. Taken together, the data presented in this dissertation shed new light on mechanisms by which alcohol promotes injury in the lung, as well as developed a tool to examine the potential extra-thoracic origins of this condition.

REFERENCES

1. Vallee BL. Alcohol in the Western World. *Sci Am* 1998;278:80-85.
2. National Highway Traffic Safety Administration Fatality Analysis Reporting System (FARS).; 2013.
3. Beier JI, Arteel GE, McClain CJ. Advances in Alcoholic Liver Disease. *Curr Gastroenterol Rep* 2011;13:56-64.
4. Guidot DM, Roman J. Chronic Ethanol Ingestion Increases Susceptibility to Acute Lung Injury: Role of Oxidative Stress and Tissue Remodeling. *Chest* 2002;122:309S-314S.
5. Adachi J, Asano M, Ueno Y, Niemela O, Ohlendieck K, Peters TJ, Preedy VR. Alcoholic Muscle Disease and Biomembrane Perturbations (Review). *J Nutr Biochem* 2003;14:616-625.
6. Crews FT, Nixon K. Mechanisms of Neurodegeneration and Regeneration in Alcoholism. *Alcohol Alcohol* 2009;44:115-127.
7. Pandol SJ, Raraty M. Pathobiology of Alcoholic Pancreatitis. *Pancreatology* 2007;7:105-114.
8. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Alcohol Consumption and Ethyl Carbamate. *IARC Monogr Eval Carcinog Risks Hum* 2010;96:3-1383.
9. US Burden of Disease Collaborators. The State of US Health, 1990-2010: Burden of Diseases, Injuries, and Risk Factors. *JAMA* 2013;310:591-608.
10. Addison T. Observations on Fatty Degeneration of the Liver. *Guy's Hosp Rep* 1836;1:485.
11. Lelbach WK. Liver Damage in Chronic Alcoholism: Results of a Clinical, Clinical-Chemical and Biopsic-Histological Study in 526 Alcoholic Patients During a Low Calorie Diet in an Open Drinking Sanatorium. *Acta Hepatosplenol* 1966;13:321-349.
12. Mann RE, Smart RG, Govoni R. The Epidemiology of Alcoholic Liver Disease. *Alcohol Res Health* 2003;27:209-219.

13. Day CP. Who Gets Alcoholic Liver Disease: Nature or Nurture? *J R Coll Physicians Lond* 2000;34:557-562.
14. Diehl AM. Liver Disease in Alcohol Abusers: Clinical Perspective. *Alcohol* 2002;27:7-11.
15. Powell WJ, Jr., Klatskin G. Duration of Survival in Patients With Laennec's Cirrhosis. Influence of Alcohol Withdrawal, and Possible Effects of Recent Changes in General Management of the Disease. *Am J Med* 1968;44:406-420.
16. La Vecchia C, Negri E, D'Avanzo B, Boyle P, Franceschi S. Medical History and Primary Liver Cancer. *Cancer Res* 1990;50:6274-6277.
17. Gogel BM, Goldstein RM, Kuhn JA, McCarty TM, Donahoe A, Glastad K. Diagnostic Evaluation of Hepatocellular Carcinoma in a Cirrhotic Liver. *Oncology (Williston Park)* 2000;14:15-20.
18. Kershaw CD. Alcoholic Lung Disease. *Alcohol Res Health* 2008;31:66-75.
19. Moss M, Bucher B, Moore FA, Moore EE, Parsons PE. The Role of Chronic Alcohol Abuse in the Development of Acute Respiratory Distress Syndrome in Adults. *JAMA* 1996;275:50-54.
20. Moss M, Burnham EL. Chronic Alcohol Abuse, Acute Respiratory Distress Syndrome, and Multiple Organ Dysfunction. *Crit Care Med* 2003;31:S207-S212.
21. Moss M, Parsons PE, Steinberg KP, Hudson LD, Guidot DM, Burnham EL, Eaton S, Cotsonis GA. Chronic Alcohol Abuse Is Associated With an Increased Incidence of Acute Respiratory Distress Syndrome and Severity of Multiple Organ Dysfunction in Patients With Septic Shock. *Crit Care Med* 2003;2003/03/11:869-877.
22. Bechara RI, Brown LA, Roman J, Joshi PC, Guidot DM. Transforming Growth Factor Beta1 Expression and Activation Is Increased in the Alcoholic Rat Lung. *Am J Respir Crit Care Med* 2004;170:188-194.
23. Bechara RI, Pelaez A, Palacio A, Joshi PC, Hart CM, Brown LA, Raynor R, Guidot DM. Angiotensin II Mediates Glutathione Depletion, Transforming Growth Factor-Beta1 Expression, and Epithelial Barrier Dysfunction in the Alcoholic Rat Lung. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L363-L370.
24. Brown LA, Harris FL, Guidot DM. Chronic Ethanol Ingestion Potentiates TNF-Alpha-Mediated Oxidative Stress and Apoptosis in Rat Type II Cells. *Am J Physiol Lung Cell Mol Physiol* 2001;281:L377-L386.

25. Krzanowski JJ, Jr., Fertel R, Matschinsky FM. Energy Metabolism in Pancreatic Islets of Rats. Studies With Tolbutamide and Hypoxia. *Diabetes* 1971;20:598-606.
26. Roman J. Extracellular Matrix and Lung Inflammation. *Immunol Res* 1996;15:163-178.
27. Burnham EL, Moss M, Ritzenthaler JD, Roman J. Increased Fibronectin Expression in Lung in the Setting of Chronic Alcohol Abuse. *Alcohol Clin Exp Res* 2007;31:675-683.
28. Holguin F, Moss I, Brown LA, Guidot DM. Chronic Ethanol Ingestion Impairs Alveolar Type II Cell Glutathione Homeostasis and Function and Predisposes to Endotoxin-Mediated Acute Edematous Lung Injury in Rats. *J Clin Invest* 1998;101:761-768.
29. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, Kuebler WM. An Official American Thoracic Society Workshop Report: Features and Measurements of Experimental Acute Lung Injury in Animals. *Am J Respir Cell Mol Biol* 2011;44:725-738.
30. Matute-Bello G, Frevert CW, Martin TR. Animal Models of Acute Lung Injury. *Am J Physiol Lung Cell Mol Physiol* 2008;295:L379-L399.
31. Hirano S. Migratory Responses of PMN After Intraperitoneal and Intratracheal Administration of Lipopolysaccharide. *Am J Physiol* 1996;270:L836-L845.
32. Reutershan J, Ley K. Bench-to-Bedside Review: Acute Respiratory Distress Syndrome - How Neutrophils Migrate into the Lung. *Crit Care* 2004;8:453-461.
33. Kabir K, Gelinas J, Chen M, Chen D, Zhang D, Luo X, Yang JH, Carter D, Rabinovici R. Characterization of a Murine Model of Endotoxin-Induced Acute Lung Injury. *Shock* 2001;17:300-303.
34. Bode CH, Kugler V, Bode JCH. Endotoxemia in Patients With Alcoholic and Non-Alcoholic Cirrhosis and in Subjects With No Evidence of Chronic Liver Disease Following Acute Alcohol Excess. *J Hepatol* 1987;4:8-14.
35. Massey VL, Poole LG, Siow DL, Torres E, Warner NL, Schmidt RH, Ritzenthaler JD, Roman J, Arteel GE. Chronic Alcohol Exposure Enhances Lipopolysaccharide-Induced Lung Injury in Mice: Potential Role of Systemic Tumor Necrosis Factor Alpha. *Alcoholism, clinical and experimental research*. (In press).
36. Arteel GE. Oxidants and Antioxidants in Alcohol-Induced Liver Disease. *Gastroenterology* 2003;124:778-790.

37. Deaciuc IV, Nikolova-Karakashian M, Fortunato F, Lee EY, Hill DB, McClain CJ. Apoptosis and Dysregulated Ceramide Metabolism in a Murine Model of Alcohol-Enhanced Lipopolysaccharide Hepatotoxicity. *Alcohol Clin Exp Res* 2000;24:1557-1565.
38. Beier JL, Luyendyk JP, Guo L, von Montfort C, Staunton DE, Arteel GE. Fibrin Accumulation Plays a Critical Role in the Sensitization to Lipopolysaccharide-Induced Liver Injury Caused by Ethanol in Mice. *Hepatology* 2009;49:1545-1553.
39. Lois M, Brown LA, Moss IM, Roman J, Guidot DM. Ethanol Ingestion Increases Activation of Matrix Metalloproteinases in Rat Lungs During Acute Endotoxemia. *Am J Respir Crit Care Med* 1999;160:1354-1360.
40. Roman J, Ritzenthaler JD, Bechara R, Brown LA, Guidot D. Ethanol Stimulates the Expression of Fibronectin in Lung Fibroblasts Via Kinase-Dependent Signals That Activate CREB. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L975-L987.
41. Enomoto N, Ikejima K, Bradford BU, Rivera CA, Kono H, Brenner DA, Thurman RG. Alcohol Causes Both Tolerance and Sensitization of Rat Kupffer Cells Via Mechanisms Dependent on Endotoxin. *Gastroenterology* 1998;115:443-451.
42. McMullen MR, Pritchard MT, Wang Q, Millward CA, Croniger CM, Nagy LE. Early Growth Response-1 Transcription Factor Is Essential for Ethanol-Induced Fatty Liver Injury in Mice. *Gastroenterology* 2005;128:2066-2076.
43. Massey VL, Beier JL, Ritzenthaler JD, Roman J, Arteel GE. Potential Role of the Gut/Liver/Lung Axis in Alcohol-Induced Tissue Pathology. *Biomolecules* 2015;5:2477-2503.
44. Poole LG, Arteel GE. Transitional Remodeling of the Hepatic Extracellular Matrix in Alcohol-Induced Liver Injury. *Biomed Res Int* 2016;2016:3162670.
45. Kruithof EK. Plasminogen Activator Inhibitors--a Review. *Enzyme* 1988;40:113-121.
46. Bergheim I, Guo L, Davis MA, Duveau I, Arteel GE. Critical Role of Plasminogen Activator Inhibitor-1 in Cholestatic Liver Injury and Fibrosis. *J Pharmacol Exp Ther* 2006;316:592-600.
47. Bergheim I, Guo L, Davis MA, Lambert JC, Beier JL, Duveau I, Luyendyk JP, Roth RA, Arteel GE. Metformin Prevents Alcohol-Induced Liver Injury in the Mouse: Critical Role of Plasminogen Activator Inhibitor-1. *Gastroenterology* 2006;130:2099-2112.

48. Idell S. Endothelium and Disordered Fibrin Turnover in the Injured Lung: Newly Recognized Pathways. *Crit Care Med* 2002;30:S274-S280.
49. Marshall BC, Sageser DS, Rao NV, Emi M, Hoidal JR. Alveolar Epithelial Cell Plasminogen Activator. Characterization and Regulation. *J Biol Chem* 1990;265:8198-8204.
50. Ware LB, Matthay MA, Parsons PE, Thompson BT, Januzzi JL, Eisner MD, The National Heart LaBIARDSCTN. Pathogenetic and Prognostic Significance of Altered Coagulation and Fibrinolysis in Acute Lung Injury/Acute Respiratory Distress Syndrome. *Crit Care Med* 2007;35:1821-1828.
51. McClintock D, Zhuo H, Wickersham N, Matthay MA, Ware LB. Biomarkers of Inflammation, Coagulation and Fibrinolysis Predict Mortality in Acute Lung Injury. *Crit Care* 2008;12:R41.
52. Sapru A, Curley MAQ, Brady S, Matthay MA, Flori H. Elevated PAI-1 Is Associated With Poor Clinical Outcomes in Pediatric Patients With Acute Lung Injury. *Intensive Care Med* 2010;36:157-163.
53. Ren W, Wang Z, Hua F, Zhu L. Plasminogen Activator Inhibitor-1 Regulates LPS-Induced TLR4/MD-2 Pathway Activation and Inflammation in Alveolar Macrophages. *Inflammation* 2015;38:384-393.
54. Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH. Bleomycin-Induced Pulmonary Fibrosis in Transgenic Mice That Either Lack or Overexpress the Murine Plasminogen Activator Inhibitor-1 Gene. *J Clin Invest* 1996;97:232-237.
55. Kwaan HC, Mazar AP, McMahon BJ. The Apparent UPA/PAI-1 Paradox in Cancer: More Than Meets the Eye. *Semin Thromb Hemost* 2013;39:382-391.
56. Chuang-Tsai S, Sisson TH, Hattori N, Tsai CG, Subbotina NM, Hanson KE, Simon RH. Reduction in Fibrotic Tissue Formation in Mice Genetically Deficient in Plasminogen Activator Inhibitor-1. *Am J Pathol* 2003;163:445-452.
57. Arndt PG, Young SK, Worthen GS. Regulation of Lipopolysaccharide-Induced Lung Inflammation by Plasminogen Activator Inhibitor-1 Through a JNK-Mediated Pathway. *J Immunol* 2005;175:4049-4059.
58. Liotta LA, Goldfarb RH, Brundage R, Siegal GP, Terranova V, Garbisa S. Effect of Plasminogen Activator (Urokinase), Plasmin, and Thrombin on Glycoprotein and Collagenous Components of Basement Membrane. *Cancer Res* 1981;41:4629-4636.

59. Mochan E, Keler T. Plasmin Degradation of Cartilage Proteoglycan. *Biochim Biophys Acta* 1984;800:312-315.
60. Mackay AR, Corbitt RH, Hartzler JL, Thorgeirsson UP. Basement Membrane Type IV Collagen Degradation: Evidence for the Involvement of a Proteolytic Cascade Independent of Metalloproteinases. *Cancer Res* 1990;50:5997-6001.
61. Ramos-DeSimone N, Hahn-Dantona E, Siple J, Nagase H, French DL, Quigley JP. Activation of Matrix Metalloproteinase-9 (MMP-9) Via a Converging Plasmin/Stromelysin-1 Cascade Enhances Tumor Cell Invasion. *J Biol Chem* 1999;274:13066-13076.
62. Brown LA, Ritzenthaler JD, Guidot DM, Roman J. Alveolar Type II Cells From Ethanol-Fed Rats Produce a Fibronectin-Enriched Extracellular Matrix That Promotes Monocyte Activation. *Alcohol* 2007;41:317-324.
63. Vial D, McKeown-Longo PJ. PAI1 Stimulates Assembly of the Fibronectin Matrix in Osteosarcoma Cells Through Crosstalk Between the $\alpha_5\beta_1$ and $\alpha_5\beta_3$ Integrins. *Journal of Cell Science* 2008;121:1661-1670.
64. Johansson A, Lundborg M, Skold CM, Lundahl J, Tornling G, Eklund A, Camner P. Functional, Morphological, and Phenotypical Differences Between Rat Alveolar and Interstitial Macrophages. *Am J Respir Cell Mol Biol* 1997;16:582-588.
65. Massey VL, Arteel GE. Acute Alcohol-Induced Liver Injury. *Frontiers in Physiology* 2012;3:1-8.
66. Antonov AS, Antonova GN, Munn DH, Mivechi N, Lucas R, Catravas JD, Verin AD. $\alpha_v\beta_3$ Integrin Regulates Macrophage Inflammatory Responses Via PI3 Kinase/Akt-Dependent NF-KB Activation. *Journal of Cell Physiology* 2010;226:464-476.
67. Lazar MH, Christensen PJ, Du M, Yu B, Subbotina NM, Hanson KE, Hansen JM, White ES, Simon RH, Sisson TH. Plasminogen Activator Inhibitor-1 Impairs Alveolar Epithelial Repair by Binding to Vitronectin. *Am J Respir Cell Mol Biol* 2004;31:672-678.
68. Arteel GE. Animal Models of Alcoholic Liver Disease. *Dig Dis* 2010;28:729-736.
69. Yuki T, Thurman RG. The Swift Increase in Alcohol Metabolism: Time Course for the Increase in Hepatic Oxygen Uptake and the Involvement of Glycolysis. *Biochem J* 1980;186:119-126.
70. Chen MM, Palmer JL, Ippolito JA, Curtis BJ, Choudhry MA, Kovacs EJ. Intoxication by Intraperitoneal Injection or Oral Gavage Equally

Potentiates Postburn Organ Damage and Inflammation. *Mediators Inflamm* 2013;2013:971481.

71. Yeligar SM, Harris FL, Hart CM, Brown LA. Glutathione Attenuates Ethanol-Induced Alveolar Macrophage Oxidative Stress and Dysfunction by Downregulating NADPH Oxidases. *Am J Physiol Lung Cell Mol Physiol* 2014;306:L429-L441.
72. Mathews S, Xu M, Wang H, Bertola A, Gao B. Animals Models of Gastrointestinal and Liver Diseases. Animal Models of Alcohol-Induced Liver Disease: Pathophysiology, Translational Relevance, and Challenges. *Am J Physiol Gastrointest Liver Physiol* 2014;306:G819-G823.
73. Best CH, Hartroft WS, Lucas CC, Ridout JH. Liver Damage Produced by Feeding Alcohol or Sugar and Its Prevention by Choline. *Br Med J* 1949;2:1001-1006.
74. DeCarli LM, Lieber CS. Fatty Liver in the Rat After Prolonged Intake of Ethanol With a Nutritionally Adequate New Liquid Diet. *J Nutr* 1967;91:331-336.
75. DeSantis DA, Ko Cw, Liu Y, Liu X, Hise AG, Nunez G, Croniger CM. Alcohol-Induced Liver Injury Is Modulated by Nlrp3 and Nlrp4 Inflammasomes in Mice. *Mediators Inflamm* 2013;2013:751374.
76. Brown LA, Harris FL, Bechara R, Guidot DM. Effect of Chronic Ethanol Ingestion on Alveolar Type II Cell: Glutathione and Inflammatory Mediator-Induced Apoptosis. *Alcohol Clin Exp Res* 2001;25:1078-1085.
77. Alli AA, Brewer EM, Montgomery DS, Ghant MS, Eaton DC, Brown LA, Helms MN. Chronic Ethanol Exposure Alters the Lung Proteome and Leads to Mitochondrial Dysfunction in Alveolar Type 2 Cells. *Am J Physiol Lung Cell Mol Physiol* 2014;306:L1026-L1035.
78. de la MH, Lieber CS, DeCarli LM, French SW, Lindros KO, Jarvelainen H, Bode C, Parlesak A, Bode JC. Models of Alcoholic Liver Disease in Rodents: a Critical Evaluation. *Alcohol Clin Exp Res* 2001;25:254S-261S.
79. Kono H, Bradford BU, Rusyn I, Fujii H, Matsumoto Y, Yin M, Thurman RG. Development of an Intragastric Enteral Model in the Mouse: Studies of Alcohol-Induced Liver Disease Using Knockout Technology. *J Hepatobiliary Pancreat Surg* 2000;7:395-400.
80. Bertola A, Mathews S, Ki SH, Wang H, Gao B. Mouse Model of Chronic and Binge Ethanol Feeding (the NIAAA Model). *Nat Protoc* 2013;8:627-637.

81. Moreau R, Jalan R, Gines P, Pavesi M, Angeli P, Cordoba J, Durand F, Gustot T, Saliba F, Domenicali M, et al. Acute-on-Chronic Liver Failure Is a Distinct Syndrome That Develops in Patients With Acute Decompensation of Cirrhosis. *Gastroenterology* 2013;144:1426-1437.
82. Michelena J, Altamirano J, Abrales JG, Affrè S, Morales-Ibanez O, Sancho-Bru P, Dominguez M, Garcia-Pagán JC, Fernández J, Arroyo V, et al. Systemic Inflammatory Response and Serum Lipopolysaccharide Levels Predict Multiple Organ Failure and Death in Alcoholic Hepatitis. *Hepatology* 2015;62:762-772.
83. Matuschak GM, Rinaldo JE, Pinsky MR, Gavalier JS, Van Thiel DH. Effect of End Stage Liver Failure on the Incidence and Resolution of Adult Respiratory Distress Syndrome. *J Crit Care* 1987;2:162-173.
84. Dizier Sp, Forel JM, Ayzac L, Richard JC, Hraiech S, Lehingue S, Loundou A, Roch A, Guerin C, Papazian L, et al. Early Hepatic Dysfunction Is Associated With a Worse Outcome in Patients Presenting With Acute Respiratory Distress Syndrome: A Post-Hoc Analysis of the ACURASYS and PROSEVA Studies. *PLoS One* 2015;10:e0144278.
85. Siore AM, Parker RE, Stecenko AA, Cuppels C, McKean M, Christman BW, Cruz-Gervis R, Brigham KL. Endotoxin-Induced Acute Lung Injury Requires Interaction With the Liver. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L769-L776.
86. Bautista AP, Skrepnik N, Niesman MR, Bagby GJ. Elimination of Macrophages by Liposome-Encapsulated Dichloromethylene Diphosphonate Suppresses the Endotoxin-Induced Priming of Kupffer Cells. *J Leukoc Biol* 1994;55:321-327.
87. Naveau S, Giraud V, Borotto E, Aubert A, Capron F, Chaput JC. Excess Weight Risk Factor for Alcoholic Liver Disease. *Hepatology* 1997;25:108-111.
88. Iyer SS, Ramirez AM, Ritzenthaler JD, Torres-Gonzalez E, Roser-Page S, Mora AL, Brigham KL, Jones DP, Roman J, Rojas M. Oxidation of Extracellular Cysteine/Cystine Redox State in Bleomycin-Induced Lung Fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L37-L45.
89. Park JK, Utsumi T, Seo YE, Deng Y, Satoh A, Saltzman WM, Iwakiri Y. Cellular Distribution of Injected PLGA-Nanoparticles in the Liver. *Nanomedicine: Nanotechnology, Biology and Medicine* 2016;12:1365-1374.
90. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat Meth* 2012;9:671-675.

91. Parsey MV, Tudor RM, Abraham E. Neutrophils Are Major Contributors to Intraparenchymal Lung IL-1 α Expression After Hemorrhage and Endotoxemia. *J Immunol* 1998;160:1007-1013.
92. Goldblum SE, Wu KM, Jay M. Lung Myeloperoxidase As a Measure of Pulmonary Leukostasis in Rabbits. *Journal of Applied Physiology* 1985;59:1978-1985.
93. von Montfort C, Beier JI, Guo L, Kaiser JP, Arteel GE. Contribution of the Sympathetic Hormone Epinephrine to the Sensitizing Effect of Ethanol on LPS-Induced Liver Damage in Mice. *Am J Physiol Gastrointest Liver Physiol* 2008;294:G1227-G1234.
94. Chen J, Mo Y, Schlueter CF, Hoyle GW. Inhibition of Chlorine-Induced Pulmonary Inflammation and Edema by Mometasone and Budesonide. *Toxicol Appl Pharmacol* 2013;272:408-413.
95. Martin DT, Steinbach JM, Liu J, Shimizu S, Kaimakliotis HZ, Wheeler MA, Hittelman AB, Saltzman WM, Weiss RM. Surface Modified Nanoparticles Enhance Transurothelial Penetration and Delivery of Survivin siRNA in Treating Bladder Cancer. *Mol Cancer Ther* 2014;13:71-81.
96. Sims LB, Curtis LT, Frieboes HB, Steinbach-Rankins JM. Enhanced Uptake and Transport of PLGA-Modified Nanoparticles in Cervical Cancer. *J Nanobiotechnology* 2016;14:33.
97. Steinbach JM, Seo YE, Saltzman WM. Cell Penetrating Peptide-Modified Poly(Lactic-Co-Glycolic Acid) Nanoparticles With Enhanced Cell Internalization. *Acta Biomater* 2016;30:49-61.
98. Substance Abuse and Mental Health Services Administration. Results From the 2012 National Survey on Drug Use and Health: Summary of National Findings. Rockville, MD: Substance Abuse and Mental Health Services Administration; 2013. HHS Publication No. (SMA) 13-4795.
99. World Health Organization. Global Status Report on Alcohol and Health.; 2014.
100. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, Amann M, Anderson HR, Andrews KG, Aryee M, et al. A Comparative Risk Assessment of Burden of Disease and Injury Attributable to 67 Risk Factors and Risk Factor Clusters in 21 Regions, 1990-2010: a Systematic Analysis for the Global Burden of Disease Study 2010. *Lancet* 2012;380:2224-2260.
101. Beier JI, Arteel GE. Alcoholic Liver Disease and the Potential Role of Plasminogen Activator Inhibitor-1 and Fibrin Metabolism. *Exp Biol Med (Maywood)* 2012;237:1-9.

102. Zarbock A, Ley K. The Role of Platelets in Acute Lung Injury (ALI). *Front Biosci* 2009;14:150-158.
103. Weyrich AS, Zimmerman GA. Platelets in Lung Biology. *Annu Rev Physiol* 2013;75:569-591.
104. Massey VL, Poole LG, Siow DL, Torres E, Warner NL, Schmidt RH, Ritzenthaler JD, Roman J, Arteel GE. Chronic Alcohol Exposure Enhances Lipopolysaccharide-Induced Lung Injury in Mice: Potential Role of Systemic Tumor Necrosis Factor-Alpha. *Alcohol Clin Exp Res* 2015;39:1978-1988.
105. Yuo A, Kitagawa S, Kasahara T, Matsushima K, Saito M, Takaku F. Stimulation and Priming of Human Neutrophils by Interleukin-8: Cooperation With Tumor Necrosis Factor and Colony-Stimulating Factors. *Blood* 1991;78:2708-2714.
106. Allen TC, Kurdowska A. Interleukin 8 and Acute Lung Injury. *Archives of Pathology & Laboratory Medicine* 2013;138:266-269.
107. Tabuchi A, Kuebler WM. Endothelium-platelet Interactions in Inflammatory Lung Disease. *Vascular Pharmacology* 2008;49:141-150.
108. Fang J, Hodivala-Dilke K, Johnson BD, Du LM, Hynes RO, White GC, Wilcox DA. Therapeutic Expression of the Platelet-Specific Integrin, α IIb β 3, in a Murine Model for Glanzmann Thrombasthenia. *Blood* 2005;106:2671-2679.
109. Perez RL, Roman J. Fibrin Enhances the Expression of IL-1 Beta by Human Peripheral Blood Mononuclear Cells. Implications in Pulmonary Inflammation. *J Immunol* 1995;154:1879-1887.
110. Pitchford SC, Yano H, Lever R, Riffo-Vasquez Y, Ciferri S, Rose MJ, Giannini S, Momi S, Spina D, O'Connor B, et al. Platelets Are Essential for Leukocyte Recruitment in Allergic Inflammation. *Journal of Allergy and Clinical Immunology* 2003;112:109-118.
111. O'Sullivan BP, Michelson AD. The Inflammatory Role of Platelets in Cystic Fibrosis. *Am J Respir Crit Care Med* 2006;173:483-490.
112. Zarbock A, Singbartl K, Ley K. Complete Reversal of Acid-Induced Acute Lung Injury by Blocking of Platelet-Neutrophil Aggregation. *J Clin Invest* 2006;116:3211-3219.
113. Kieffmann R, Heckel K, Schenkat S, Dörger M, Goetz AE. Role of P-Selectin in Platelet Sequestration in Pulmonary Capillaries During Endotoxemia. *Journal of Vascular Research* 2006;43:473-481.

114. Brass LF. Thrombin and Platelet Activation. *Chest* 2003;124:18S-25S.
115. van Holten TC, Bleijerveld OB, Wijten P, de Groot PG, Heck AJR, Barendrecht AD, Merkx TH, Scholten A, Roest M. Quantitative Proteomics Analysis Reveals Similar Release Profiles Following Specific PAR-1 or PAR-4 Stimulation of Platelets. *Cardiovasc Res* 2014;103:140-146.
116. Sullivan BP, Kassel KM, Jone A, Flick MJ, Luyendyk JP. Fibrin(Ogen)-Independent Role of Plasminogen Activators in Acetaminophen-Induced Liver Injury. *Am J Pathol* 2012;180:2321-2329.
117. Bajt ML, Yan HM, Farhood A, Jaeschke H. Plasminogen Activator Inhibitor-1 Limits Liver Injury and Facilitates Regeneration After Acetaminophen Overdose. *Toxicol Sci* 2008;104:419-427.
118. Beier JI, Guo L, Joshi-Barve S, Ritzenthaler JD, Roman J, Arteel GE. Fibrin-Mediated Integrin Signaling Plays a Critical Role in Hepatic Regeneration After Partial Hepatectomy in Mice. *Annals of Hepatology*. (In press).
119. von Montfort C, Beier JI, Kaiser JP, Guo L, Joshi-Barve S, Pritchard MT, States JC, Arteel GE. PAI-1 Plays a Protective Role in CCl4-Induced Hepatic Fibrosis in Mice: Role of Hepatocyte Division. *Am J Physiol Gastrointest Liver Physiol* 2010;298:G657-66.
120. Hua F, Ren W, Zhu L. Plasminogen Activator Inhibitor Type-1 Deficiency Exaggerates LPS-Induced Acute Lung Injury Through Enhancing Toll-Like Receptor 4 Signaling Pathway. *Blood Coagulation & Fibrinolysis* 2011;22.
121. Kamholz SL. Wine, Spirits and the Lung: Good, Bad or Indifferent? *Trans Am Clin Climatol Assoc* 2006;117:129-145.
122. Shults JA, Curtis BJ, Boe DM, Ramirez L, Kovacs EJ. Ethanol Intoxication Prolongs Post-Burn Pulmonary Inflammation: Role of Alveolar Macrophages. *J Leukocyte Biol* 2016;100:1037-1045.
123. Yeligar SM, Chen MM, Kovacs EJ, Sisson JH, Burnham EL, Brown LA. Alcohol and Lung Injury and Immunity. *Alcohol* 2016;55:51-59.
124. Mathews S, Xu M, Wang H, Bertola A, Gao B. Animals Models of Gastrointestinal and Liver Diseases. Animal Models of Alcohol-Induced Liver Disease: Pathophysiology, Translational Relevance, and Challenges. *Am J Physiol Gastrointest Liver Physiol* 2014;306:G819-G823.
125. Joshi PC, Guidot DM. The Alcoholic Lung: Epidemiology, Pathophysiology, and Potential Therapies. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L813-L823.

126. Samokhvalov AV, Irving HM, Rehm J. Alcohol Consumption As a Risk Factor for Pneumonia: a Systematic Review and Meta-Analysis. *Epidemiol Infect* 2010;138:1789-1795.
127. Thakur L, Kojicic M, Thakur SJ, Pieper MS, Kashyap R, Trillo-Alvarez CA, Javier F, Cartin-Ceba R, Gajic O. Alcohol Consumption and Development of Acute Respiratory Distress Syndrome: a Population-Based Study. *Int J Environ Res Public Health* 2009;6:2426-2435.
128. Lieber CS, Jones DP, DeCarli LM. Effects of Prolonged Ethanol Intake: Production of Fatty Liver Despite Adequate Diets. *J Clin Invest* 1965;44:1009-1021.
129. Urso T, Gavaler JS, Van Thiel DH. Blood Ethanol Levels in Sober Alcohol Users Seen in an Emergency Room. *Life Sci* 1981;28:1053-1056.
130. Goldman MS. To Drink or Not to Drink: an Experimental Analysis of Group Drinking Decisions by Four Alcoholics. *Am J Psychiatry* 1974;131:1123-1130.
131. Wilkin RJ, Lalor PF, Parker R, Newsome PN. Murine Models of Acute Alcoholic Hepatitis and Their Relevance to Human Disease. *Am J Pathol* 2016;186:748-760.
132. Hingson RW, Zha W, White AM. Drinking Beyond the Binge Threshold: Predictors, Consequences, and Changes in the U.S. *American Journal of Preventive Medicine* 2017;52:717-727.
133. Parrott PD, Mitzner W. Airway-Parenchymal Interdependence. *Compr Physiol* 2012;2:1921-1935.
134. Myou S, Fujimura M, Nishi K, Matsuda M, Ohka T, Matsuda T. Potentiating Effect of Inhaled Acetaldehyde on Bronchial Responsiveness to Methacholine in Asthmatic Subjects. *Thorax* 1994;49:644-648.
135. Gong H, Jr., Tashkin DP, Calvarese BM. Alcohol-Induced Bronchospasm in an Asthmatic Patient: Pharmacologic Evaluation of the Mechanism. *Chest* 1981;80:167-173.
136. Ayres J, Ancic P, Clark TJ. Airways Responses to Oral Ethanol in Normal Subjects and in Patients With Asthma. *J R Soc Med* 1982;75:699-704.
137. Ayres JG, Clark TJ. Intravenous Ethanol Can Provide Bronchodilatation in Asthma. *Clin Sci (Lond)* 1983;64:555-557.
138. Oldenburg PJ, Poole JA, Sisson JH. Alcohol Reduces Airway Hyperresponsiveness (AHR) and Allergic Airway Inflammation in Mice. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L308.

139. Brandl K, Schnabl B. Is Intestinal Inflammation Linking Dysbiosis to Gut Barrier Dysfunction During Liver Disease? *Expert Rev Gastroenterol Hepatol* 2015;9:1069-1076.
140. Matuschak GM. Lung-Liver Interactions in Sepsis and Multiple Organ Failure Syndrome. *Clin Chest Med* 1996;17:83-98.
141. Matuschak GM, Mattingly ME, Tredway TL, Lechner AJ. Liver-Lung Interactions During E. Coli Endotoxemia. TNF-Alpha:Leukotriene Axis. *Am J Respir Crit Care Med* 1994;149:41-49.
142. Matuschak GM, Pinsky MR, Klein EC, Van Thiel DH, Rinaldo JE. Effects of D-Galactosamine-Induced Acute Liver Injury on Mortality and Pulmonary Responses to Escherichia Coli Lipopolysaccharide. Modulation by Arachidonic Acid Metabolites. *Am Rev Respir Dis* 1990;141:1296-1306.
143. Greenhalgh SN, Conroy KP, Henderson NC. Cre-Activity in the Liver: Transgenic Approaches to Targeting Hepatic Nonparenchymal Cells. *Hepatology* 2015;61:2091-2099.
144. van Rooijen N, van Kesteren-Hendrikx E. CLODRONATE LIPOSOMES: PERSPECTIVES IN RESEARCH AND THERAPEUTICS. *Journal of Liposome Research* 2002;12:81-94.
145. Danhier F, Ansorena E, Silva JM, Coco R, Le BA, Preat V. PLGA-Based Nanoparticles: an Overview of Biomedical Applications. *J Control Release* 2012;161:505-522.
146. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT. Systemic LPS Causes Chronic Neuroinflammation and Progressive Neurodegeneration. *GLIA* 2007;55:453-462.
147. Folch-Puy E. Importance of the Liver in Systemic Complications Associated With Acute Pancreatitis: the Role of Kupffer Cells. *J Pathol* 2007;211:383-388.
148. Yang CY, Chen JB, Tsai TF, Tsai YC, Tsai CY, Liang PH, Hsu TL, Wu CY, Netea MG, Wong CH, et al. CLEC4F Is an Inducible C-Type Lectin in F4/80-Positive Cells and Is Involved in Alpha-Galactosylceramide Presentation in Liver. *PLoS One* 2013;8:e65070.
149. Patterson EK, Yao LJ, Ramic N, Lewis JF, Cepinskas G, McCaig L, Veldhuizen RAW, Yamashita CM. Lung-Derived Mediators Induce Cytokine Production in Downstream Organs Via an NF- κ B-Dependent Mechanism. *Mediators Inflamm* 2013;2013:586895.

150. Truscott EA, McCaig LA, Yao LJ, Veldhuizen RAW, Lewis JF. Surfactant Protein-A Reduces Translocation of Mediators From the Lung into the Circulation. *Experimental Lung Research* 2010;36:431-439.
151. MacLaren R, Stringer KA. Emerging Role of Anticoagulants and Fibrinolytics in the Treatment of Acute Respiratory Distress Syndrome. *Pharmacotherapy* 2007;27:860-873.
152. Bertola A, Park O, Gao B. Chronic Plus Binge Ethanol Feeding Synergistically Induces Neutrophil Infiltration and Liver Injury: A Critical Role for E-Selectin. *Hepatology* 2013:10.
153. Qin L, He J, Hanes RN, Pluzarev O, Hong JS, Crews FT. Increased Systemic and Brain Cytokine Production and Neuroinflammation by Endotoxin Following Ethanol Treatment. *J Neuroinflammation* 2008;5:10.
154. GLAS GJ, VAN DER SLUIJS KF, SCHULTZ MJ, HOFSTRA J-JH, van der Poll T, Levi M. Bronchoalveolar Hemostasis in Lung Injury and Acute Respiratory Distress Syndrome. *Journal of Thrombosis and Haemostasis* 2013;11:17-25.
155. Wang M, You Q, Lor K, Chen F, Gao B, Ju C. Chronic Alcohol Ingestion Modulates Hepatic Macrophage Populations and Functions in Mice. *J Leukoc Biol* 2014;96:657-665.
156. Ju C, Mandrekar P. Macrophages and Alcohol-Related Liver Inflammation. *Alcohol Res* 2015;37:251-262.
157. Sullivan BP, Wang R, Tawfik O, Luyendyk JP. Protective and Damaging Effects of Platelets in Acute Cholestatic Liver Injury Revealed by Depletion and Inhibition Strategies. *Toxicol Sci* 2010;115:286-294.
158. Quinn MJ, Plow EF, Topol EJ. Platelet Glycoprotein IIb/IIIa Inhibitors. *Circulation* 2002;106:379.
159. Joshi N, Kopec AK, Ray JL, Luyendyk JP. Inhibition of PAR-4 and P2Y12 Receptor-Mediated Platelet Activation Produces Distinct Hepatic Pathologies in Experimental Xenobiotic-Induced Cholestatic Liver Disease. *Toxicol* 2016;365:9-16.
160. Eguchi H, McCuskey PA, McCuskey RS. Kupffer Cell Activity and Hepatic Microvascular Events After Acute Ethanol Ingestion in Mice. *Hepatology* 1991;13:751-757.
161. Peck-Radosavljevic M. Thrombocytopenia in Chronic Liver Disease. *Liver International* 2016:n/a.

162. Collins RA, Gualano RC, Zosky GR, Atkins CL, Turner DJ, Colasurdo GN, Sly PD. Hyperresponsiveness to Inhaled but Not Intravenous Methacholine During Acute Respiratory Syncytial Virus Infection in Mice. *Respir Res* 2005;6:142.
163. Baughman RP, Roselle GA. Surfactant Deficiency With Decreased Opsonic Activity in a Guinea Pig Model of Alcoholism. *Alcoholism: Clinical and Experimental Research* 1987;11:261-264.
164. Echaide M, Autilio C, Arroyo R, Perez-Gil J. Restoring Pulmonary Surfactant Membranes and Films at the Respiratory Surface. *Biochimica et Biophysica Acta (BBA) - Biomembranes*.
165. Bowler RP, Crapo JD. Oxidative Stress in Allergic Respiratory Diseases. *Journal of Allergy and Clinical Immunology* 2002;110:349-356.
166. Ritzenthaler JD, Roser-Page S, Guidot DM, Roman J. Nicotinic Acetylcholine Receptors Are Sensors for Ethanol in Lung Fibroblasts. *Alcohol Clin Exp Res* 2013;37:914-923.
167. O'Halloran EB, Curtis BJ, Afshar M, Chen MM, Kovacs EJ, Burnham EL. Alveolar Macrophage Inflammatory Mediator Expression Is Elevated in the Setting of Alcohol Use Disorders. *Alcohol* 2016;50:43-50.
168. Burnham EL, Kovacs EJ, Davis CS. Pulmonary Cytokine Composition Differs in the Setting of Alcohol Use Disorders and Cigarette Smoking. *Am J Physiol Lung Cell Mol Physiol* 2013;304:L873-L882.
169. Idzko M, Pitchford S, Page C. Role of Platelets in Allergic Airway Inflammation. *Journal of Allergy and Clinical Immunology* 2015;135:1416-1423.
170. Canning BJ, Fischer A. Neural Regulation of Airway Smooth Muscle Tone. *Respiration Physiology* 2001;125:113-127.
171. Canning BJ. Role of Nerves in Asthmatic Inflammation and Potential Influence of Gastroesophageal Reflux Disease. *The American Journal of Medicine* 2001;111:13-17.

ABBREVIATIONS

AH	Alcoholic hepatitis
ALD	alcoholic liver disease
ALI	Acute lung injury
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
ARDS	Acute respiratory distress syndrome
AST	Aspartate aminotransferase
AUD	Alcohol use disorder
BAC	Blood alcohol content
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
C	Compliance
CAE	Chloroacetate esterase
CCL5	C-C chemokine ligand-1
CD41	Cluster of differentiation-41
CD68	Cluster of differentiation-68
Cre	Cyclization recombinase
CXCL1	C-X-C chemokine ligand-1
CXCL15	C-X-C chemokine ligand-15
CXCL2	C-X-C chemokine ligand-2

DALY	Disability-adjusted life year
DCM	Dichloromethane
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
G	Tissue damping
H	Tissue elastance
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
ICAM-1	Intracellular adhesion molecule-1
IL-1 β	Interleukin-1 beta
IL-22	Interleukin-22
IL-23	Interleukin-23
IL-6	Interleukin-6
KC	Keratinocyte chemotractant
Loxp	Locus of X(cross)-over in P1
LPS	Lipopolysaccharide
MCP-1	Monocyte chemotactic protein-1
mG	Membrane-targeted enhanced green fluorescent protein
a	Macrophage inflammatory protein-2

MMP	matrix metalloprotease
mT	Membrane-targeted tandem-dimer Tomato
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NP	Nanoparticle
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease-activated receptor
PBS	Phosphate-buffered saline
PECAM	Platelet-endothelial cell adhesion molecule
PFA	Paraformaldehyde
PLGA	Poly(lactic-co-glycolic acid)
PMN	Polymorphonuclear leukocyte
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RANTES	Regulated on activation, normal T-cell expressed and secreted
RGD	arginine-glycine-aspartic acid domain
R _n	Newtonian resistance
R _{rs}	Resistance
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
TACE	TNF α -converting enzyme
TAT	Thrombin anti-thrombin
TNF α	Tumor necrosis factor-alpha
tPA	Tissue-type plasminogen activator

uPA	Urokinase-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
WT	Wildtype

CURRICULUM VITAE

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EDUCATION

- | | |
|-----------|--|
| 2013-2015 | M.S. in Pharmacology and Toxicology, University of Louisville, Louisville KY |
| 2009-2013 | B.S. in Biology, Minor in Spanish, University of Louisville, Louisville KY |

PROFESSIONAL EXPERIENCE

- | | |
|--------------|---|
| 2013-present | Graduate Research Assistant, University of Louisville, Department of Pharmacology and Toxicology |
| 2013 | NIH R25 Cancer Education Program Trainee, University of Louisville |
| 2012-2013 | Undergraduate Research Assistant, University of Louisville, Department of Pharmacology and Toxicology |
| 2012 | Summer Research Opportunities Program Trainee, University of Louisville |

PROFESSIONAL MEMBERSHIPS AND ACTIVITIES

- | | |
|--------------|--|
| 2016-present | Society of Toxicology, Student Member |
| 2016-present | Association for Women in Science, Student Member |
| 2015-present | Society for Leukocyte Biology, Student Member |
| 2014-present | Ohio Valley Society of Toxicology |
| 2014-present | University of Louisville Science Policy and Outreach Group |
| 2013-present | University of Louisville Alcohol Research Center |

2013-2014	University of Louisville Woodcock Society. This is prestigious honors society comprising the top 10% of graduates who demonstrate outstanding scholarship.
2011-2013	Golden Key International Honors Society

HONORS AND AWARDS

03/2017	University of Louisville Graduate Student Regional Research Conference: Best Poster
10/2016	Ohio Valley Society of Toxicology Ph.D. Student Platform Contest: Travel Award
10/2015	Research!Louisville Doctoral Basic Science Graduate Student Award: First Place
09/2015	School of Interdisciplinary and Graduate Studies Travel Award
09/2015	Alcohol and Immunology Research Interest Group Travel Award
11/2014	School of Interdisciplinary and Graduate Studies Travel Award
05/2013	David G. Smith Memorial Award for Excellence in Undergraduate Research
05/2013	Magna Cum Laude
05/2013	University of Louisville Honors Scholar
2009-2013	Dean's List, University of Louisville
2009-2013	Hallmark Scholarship, University of Louisville
2009	International Baccalaureate Diploma

SERVICE

Community Service

2017	Judge, Louisville Regional Science and Engineering Fair. Louisville, KY
2016	Judge, Louisville Regional Science and Engineering Fair. Louisville, KY
2015	Judge, Our Lady of Lourdes Sixth and Seventh Grade Science Fair. Louisville, KY
2012	Mentor, The College Prep Program, Western Middle School. Louisville, KY. The goal of this program is to provide gifted middle school students in underserved areas with tutoring services and mentoring to encourage them to pursue higher education.

2012	Administrative assistant, La Casita Center. Louisville, KY. The mission of this center is to empower women and families in the Hispanic community by meeting basic needs (e.g., food, housing, clothing), providing educational services, and building support networks.
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EDUCATION ACTIVITIES

Lectures/labs

2015	Lecturer, Biology 395: Basic Pharmacology, University of Louisville Dept. of Biology
2014	Mentor, Medical Pharmacology Patient Simulator Session
2012	Undergraduate Teaching Assistant, Biology 244: Principles of Biology Lab, University of Louisville Dept. of Biology

Professional Development

2011-2012	Board of Overseers Mentoring Program, University of Louisville
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LEADERSHIP

2016-present	President, Pharmacology and Toxicology Graduate Student Organization, University of Louisville
2016-present	Representative to the Graduate Student Council, University of Louisville
2016-present	Student Representative to the Student Affairs Committee, Dept. of Pharmacology and Toxicology, University of Louisville
2015-2016	Vice President, Pharmacology and Toxicology Graduate Student Organization, University of Louisville
2013-present	Class Representative, Department of Pharmacology and Toxicology, University of Louisville
2010-2013	Captain, University of Louisville Polo Club
2010-2012	President, University of Louisville Polo Club

FUNDING

08/2017	NIEHS NRSA Institutional Training Grant (T32) Postdoctoral Fellowship, Michigan State University, Institute for Integrative Toxicology
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2016-present	NIEHS NRSA Institutional Training Grant (T32) Predoctoral Fellowship, University of Louisville, Department of Pharmacology and Toxicology
2013-2015	Graduate Research Fellowship, Integrated Programs in Biomedical Sciences, University of Louisville

ABSTRACTS AND PRESENTATIONS

ORAL PRESENTATIONS

Local/Regional

1. 10/2016. Platform Presentation, Ohio Valley Society of Toxicology Annual Meeting, Indianapolis, IN
2. 07/2015. Research Seminar, University of Louisville, Seminar in Pharmacology and Toxicology, Louisville, KY.
3. 06/2015. Platform Presentation, Ohio Valley Society of Toxicology Summer Student and Postdoc Meeting, Cincinnati, OH
4. 03/2015. Research Seminar, University of Louisville, University of Louisville Alcohol Research Center, Louisville, KY.
5. 03/2014. Research Seminar, University of Louisville, Seminar in Pharmacology and Toxicology, Louisville, KY.

National

1. 02/2017. Research Seminar, University of New Mexico Department of Pharmaceutical Sciences, Albuquerque, NM.
2. 02/2017. Research Seminar, University of Arizona Department of Pharmacology and Toxicology, Tucson, AZ.
3. 09/2015. Research Symposium, Society for Leukocyte Biology National Meeting, Raleigh NC

POSTERS

Local/Regional

1. 10/2016 **Poole LG**, Beier JI, Torres E, Mohamed A, Warner NL, Dolin CE, Nguyen-Ho CT, Ritzenthaler JD, Roman J, Arteel GE. Acute-on-chronic alcohol exposure using the 'NIAAA model' concomitantly damages the liver and lung. Research!Louisville, Louisville, KY.
2. 06/2016 **Poole LG**, Beier JI, Torres E, Mohamed A, Warner NL, Dolin CE, Nguyen-Ho CT, Ritzenthaler JD, Roman J, Arteel GE. Acute-on-chronic alcohol exposure using the 'NIAAA model' concomitantly damages the liver and lung. Ohio Valley Society of Toxicology Summer Meeting, Cincinnati OH
3. 11/2015. **Poole LG**, Massey VL, Torres E, Siow DL, Warner NL, Lang AL, Dolin CE, Luyendyk JP, Ritzenthaler J, Roman J, Arteel GE. Chronic ethanol exposure sensitizes the lung in a mouse model of endotoxemia-induced acute lung injury: potential role of plasminogen activator inhibitor-1. Research!Louisville, Louisville, KY
4. 09/2014. **Poole LG**, Massey VL, Dolin CE, Siow DS, Merchant ML, Beier JI, Roman J, Arteel GE. Ethanol-induced changes in the hepatic ECM directly enhance the pro-inflammatory response of macrophages. Ohio Valley Society of Toxicology Fall Meeting, Dayton OH
5. 09/2014. **Poole LG**, Massey VL, Dolin CE, Siow DS, Merchant ML, Beier JI, Roman J, Arteel GE. Ethanol-induced changes in the hepatic ECM directly enhance the pro-inflammatory response of macrophages. Research!Louisville, Louisville KY
6. 09/2013. **Poole LG**, Jokinen JD, Massey VL, Beier JI, Arteel GE. Sinusoidal endothelial cell-derived extracellular matrix regulates basal and stimulated macrophage activation. Research!Louisville, Louisville KY
7. 07/2012. **Poole LG**, Jokinen JD, Massey VL, Beier JI, Arteel GE. Investigating the role of sinusoidal endothelial cell-derived extracellular matrix in the hepatic macrophage inflammatory response. Summer Research Opportunities Undergraduate Research Symposium, Louisville KY

National

1. 11/2016. **Poole LG**, Beier JI, Torres-González E, Anwar-Mohamed A, Warner NL, Dolin CE, Nguyen-Ho CT, Roman J, Arteel GE. Acute-on-chronic alcohol exposure using the 'NIAAA model' concomitantly damages the liver and lung. The Liver Meeting, Boston MA
2. 05/2016. **Poole LG**, Massey VL, Torres-González E, Warner NL, Siow DL, Luyendyk JP, Dolin CE, Ritzenthaler J, Roman J, Arteel GE. Plasminogen activator inhibitor-1 plays a critical role in alcohol-enhanced acute lung injury. American Thoracic Society, San Francisco, CA
3. 11/2015. **Poole LG**, Massey VL, Torres-Gonzales E, Falkner KC, Siow DL, Warner NL, Schmidt RH, Ritzenthaler JD, Roman J, Arteel GE. TNF α mediates

the liver:lung axis in alcohol-enhanced acute lung injury in mice. The Liver Meeting, San Francisco CA

4. 09/2015. **Poole LG**, Massey VL, Torres E, Siow DL, Warner NL, Lang AL, Dolin CE, Ritzenthaler J, Roman J, Arteel GE. Chronic ethanol exposure sensitizes the lung in a mouse model of endotoxemia-induced acute lung injury: potential role of plasminogen activator inhibitor-1. Society for Leukocyte Biology Annual Meeting, Raleigh NC

ABSTRACTS

National

1. **Poole LG**, Beier JI, Torres-González E, Anwar-Mohamed A, Warner NL, Dolin CE, Nguyen-Ho CT, Roman J, Arteel GE. Acute-on-chronic alcohol exposure using the 'NIAAA model' concomitantly damages the liver and lung. (2016) *Hepatology* 64 S1 (1221).
2. **Poole LG**, Massey VL, Torres-Gonzalez E, Siow DL, Warner NL, Luyendyk JP, Ritzenthaler JD, Roman J, Arteel GE. Plasminogen Activator Inhibitor-1 (PAI-1) Plays a Critical Role in Alcohol-Enhanced Acute Lung Injury [abstract]. *Am J Respir Crit Care Med* 2016:A2647.
3. Lang AL, Kaelin BR, Yeo H, Hudson SV, McKenzie CM, Sharp CN, **Poole LG**, Arteel GE, and Beier JI (2016) Critical Role of Mammalian Target of Rapamycin (mTor) in Liver Damage Caused by VC Metabolites in Mice. *The Toxicologist. Supplement to Toxicological Sciences* 150(1):231.
4. **Poole LG**, Massey VL, Torres E, Falkner KC, Siow DL, Warner NL, Schmidt RH, Ritzenthaler JD, Roman J, Arteel GE (2015) TNF α mediates the liver:lung axis in alcohol-enhanced acute lung injury in mice. *Hepatology* 62 (S1) 2142
5. Dolin CE, Massey VL, **Poole LG**, Siow DL, Merchant ML, Wilkey DW, Arteel GE (2015) The hepatic "matrisome" responds dynamically to inflammatory injury: proteomic characterization of the transitional ECM changes in the liver. *Hepatology* 62 (S1) 587
6. **Poole LG**, Massey VL, Torres E, Siow DL, Warner NL, Lang AL, Dolin CE, Ritzenthaler J, Roman J, Arteel GE. (2015) Chronic ethanol exposure sensitizes the lung in a mouse model of endotoxemia-induced acute lung injury: potential role of plasminogen activator inhibitor-1. Society for Leukocyte Biology Annual Meeting. Raleigh NC
7. Dolin CE, Massey VL, **Poole LG**, Siow DL, Merchant ML, Wilkey DW, Arteel GE. (2015) The hepatic 'matrisome' responds dynamically to stress: novel characterization of the ECM proteome. Society of Toxicology Annual Meeting. San Diego, CA
8. Bushau A.M., Anders L.C., Douglas A.D., Joshi-Barve S., **Poole L.G.**, Massey V.L., Falkner K.C., Cave M., McClain C.J., Beier J.I. Mechanistic Insight into

Vinyl Chloride-Induced Liver Injury. Society of Toxicology, March 22-26, 2015, San Diego, CA.

9. Massey VL, **Poole LG**, Torres E, Dolin C, Merchant ML, Ritzenthaler J, Roman J, Arteel GE. (2014) Characterization of the liver:lung axis in alcohol-induced lung damage. *Hepatology* 59 (S1) 616

Local/Regional

1. **Poole LG**, Beier JI, Torres-González E, Anwar-Mohamed A, Warner NL, Dolin CE, Nguyen-Ho CT, Roman J, Arteel GE. (2016). Concomitant Liver and Lung Injury in the "NIAAA Model" of Chronic + Binge Alcohol Exposure. Ohio Valley Society of Toxicology Annual Meeting, Indianapolis, IN.
2. **Poole LG**, Beier JI, Torres-González E, Anwar-Mohamed A, Warner NL, Dolin CE, Nguyen-Ho CT, Roman J, Arteel GE. (2016). Acute-on-chronic alcohol exposure using the 'NIAAA model' concomitantly damages the liver and lung. Research!Louisville, Louisville, KY.
3. Hudson SV, Dolin CE, **Poole LG**, Massey VL, Wilkey DW, Merchant ML, Frieboes H, Arteel GE. (2016). Modeling Binding Kinetics of Integrin Mediators of EtOH-enhanced LPS Liver Injury. Research!Louisville, Louisville, KY.
4. Young JL, **Poole LG**, Nguyen-Ho CT, Arteel GE. (2016) Exploring the Impact of Chromium Exposure on Macrophage Polarization: Implications for the Innate Immune Response. Research!Louisville, Louisville, KY.
5. **Poole LG**, Beier JI, Torres-González E, Anwar-Mohamed A, Warner NL, Dolin CE, Nguyen-Ho CT, Roman J, Arteel GE. (2016). Acute-on-chronic alcohol exposure using the 'NIAAA model' concomitantly damages the liver and lung. Ohio Valley Society of Toxicology Summer Meeting, Cincinnati, OH.
6. **Poole LG**, Massey VL, Torres E, Siow DL, Ritzenthaler J, Roman J, Arteel GE (2015) Chronic ethanol exposure sensitizes the lung in a mouse model of sepsis-induced acute lung injury: potential role of plasminogen activator inhibitor-1. Ohio Valley Society of Toxicology Summer Meeting
7. **Poole LG**, Massey VL, Dolin CE, Siow DS, Merchant ML, Beier JI, Roman J, Arteel GE. 2014. Ethanol-induced changes in the hepatic ECM directly enhance the pro-inflammatory response of macrophages. Ohio Valley Society of Toxicology Fall Meeting, Dayton OH
8. Dolin CE, Massey VL, **Poole LG**, Siow DL, Merchant ML, Wilkey DW, Arteel GE. (2014) The hepatic 'matrisome' responds dynamically to stress: novel characterization of the ECM proteome. Research!Louisville, Louisville, KY
9. Bushau A.M., Anders L.C., Douglas A.D., Joshi-Barve S., **Poole L.G.**, Massey V.L., Falkner K.C., Cave M., McClain C.J., Beier J.I. (2014). Mechanistic Insight into Vinyl Chloride-Induced Liver Injury. Research!Louisville, Louisville KY
10. **Poole LG**, Jokinen JD, Massey VL, Beier JI, Arteel GE. 2013. Sinusoidal endothelial cell-derived extracellular matrix regulates basal and stimulated macrophage activation. Research!Louisville, Louisville KY

11. Massey VL, **Poole LG**, Schmidt RH, Arteel GE (2012) Integrin $\beta 3^{-/-}$ mice are protected against enhanced alcohol-induced liver injury. Research!Louisville Louisville, KY
12. **Poole LG**, Jokinen JD, Massey VL, Beier JI, Arteel GE. 2012. Investigating the role of sinusoidal endothelial cell-derived extracellular matrix in the hepatic macrophage inflammatory response. Summer Research Opportunities Undergraduate Research Symposium, Louisville KY

PUBLICATIONS

Papers

1. **Poole LG**, Beier JI, Steinbach-Rankins JM, Young JL, Hudson SV, Roman J, Arteel GE. A novel, nanoparticle-based approach to selectively genetically modify hepatic macrophages. *In preparation*.
2. **Poole LG**, Beier JI, Torres-González E, Nguyen-Ho CT, Schlueter CF, Hoyle GW, Warner NL, Dolin CE, Roman J, Arteel GE. Acute-on-chronic alcohol exposure promotes pulmonary inflammation and affects lung mechanics. *In preparation*.
3. **Poole LG**, Dolin CE, Arteel GE. Organ-organ crosstalk in alcoholic liver disease. *In preparation*.
4. **Poole LG**, Massey VL, Siow DL, Torres-González E, Warner NL, Ritzenthaler JD, Roman J, Arteel GE. Plasminogen activator inhibitor-1 plays a critical role in alcohol-enhanced acute lung injury. *American Journal of Respiratory Cell and Molecular Biology* 2017 April 26 Epub ahead of print.
5. Massey VL, Dolin CE, **Poole LG**, Wilkey D, Merchant ML, Torres E, Roman J, Arteel GE. The hepatic 'matrisome' responds dynamically to stress: novel characterization of the ECM proteome. *Hepatology* 2017 Mar 65(3): 969-982
6. **Poole LG**, Arteel GE. Transitional Remodeling of the Hepatic Extracellular Matrix in Alcohol-Induced Liver Injury. *Biomed Research International*. (2016) Vol 2016, Article ID 3162670, 10 pages.
7. Cannon AR, Morris NL, Hammer AM, Curtis B, Remick DG, Yeligar SM, **Poole LG**, Burnham EL, Wyatt TA, Molina PE, So-Armah K, Cisneros T, Wang G, Lang CH, Mandrekar P, Kovacs EJ, Choudhry MA. Alcohol and Inflammatory Responses: Highlights of the 2015 Alcohol and Immunology Research Interest Group (AIRIG) Meeting. *Alcohol*. 2016 Aug; 54: 73-7
8. Massey VL, **Poole LG**, Siow DL, Torres E, Warner NL, Schmidt RH, Ritzenthaler JD, Roman J, Arteel GE. Chronic alcohol exposure enhances lipopolysaccharide-induced lung injury in mice: potential role of systemic tumor necrosis factor alpha. *Alcoholism: Clinical and Experimental Research*. 2015 Oct; 39(10): 1978-88