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Chronic ethanol exposure sensitizes the lung in a mouse model of endotoxemia-induced acute lung injury : potential role of plasminogen activator inhibitor-1.

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CHRONIC ETHANOL EXPOSURE SENSITIZES THE LUNG

IN A MOUSE MODEL OF ENDOTOXEMIA-INDUCED ACUTE LUNG INJURY:

POTENTIAL ROLE OF PLASMINOGEN ACTIVATOR INHIBITOR-1

By

Lauren G. Poole

B.S. University of Louisville, 2013

A Thesis

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Thesis Approved on

July 22, 2015

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DEDICATION

This thesis is dedicated to my parents

Janet Wolff Weiss

And

Michael Poole

for their constant encouragement and support

In all my endeavors

ACKNOWLEDGMENTS

Last but not least, I would like to thank my friends and family for their constant support: David Hardy for his endless patience, support, and for believing in me when I didn't believe in myself, my sister and role model, Meghan Poole, and of course, my parents Janet Wolff Weiss and Michael Poole for always pushing me to my highest potential.

ABSTRACT

CHRONIC ETHANOL EXPOSURE SENSITIZES THE LUNG
IN A MOUSE MODEL OF ENDOTOXEMIA-INDUCED ACUTE LUNG INJURY:
POTENTIAL ROLE OF PLASMINOGEN ACTIVATOR INHIBITOR-1

Lauren G. Poole

July 22, 2015

The goal of this project is to characterize a new mouse model of alcohol-enhanced acute lung injury (ALI) and to determine the role of plasminogen activator inhibitor-1 (PAI-1) in this model. Male mice (WT and PAI-1$^{-/-}$) were exposed to ethanol-containing Lieber-DeCarli diet or pair-fed control diet for 6 weeks; some animals were administered intraperitoneal lipopolysaccharide (LPS) prior to sacrifice. Chronic alcohol feeding enhanced induction of the chemokines MIP-2 and KC (murine IL-8 homologues) after LPS injection in wild type animals. This enhanced chemokine expression did not correlate with enhanced pulmonary neutrophil infiltration, however animals exposed to chronic ethanol showed sustained alveolar septal thickening and enhanced 4-HNE staining, indicative of inflammatory damage. Septal thickening was completely attenuated in PAI-1$^{-/-}$ animals. This work has developed a new mouse model which can be used to

elucidate the mechanisms of alcohol-enhanced ALI. A potential role of PAI-1 in

alcohol-enhanced ALI has also been identified.

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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 (1). Moreover, over 17 million Americans have been diagnosed with an alcohol use disorder (1). 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 (2), making alcohol the fifth leading risk factor for premature death and disability worldwide (3). The medical expenses for alcohol-related disease, combined with a loss in productivity, are estimated to cost the U.S. government around 223.5 billion dollars annually (4). Outside of lifestyle modification and treatment of secondary sequelae (e.g., decompensation in liver disease), treatment options to halt or reverse alcohol-related organ damage are limited. Moreover, despite increased understanding of alcohol dependence, over 90% of alcoholics will relapse at least once over a four year treatment period (5), meaning that the treatment of alcoholism is not always a viable option for preventing the negative health effects associated with alcohol abuse; this makes treatment of alcohol-related pathophysiology a critical target for investigation. Therefore, there is an urgent need to determine the mechanisms leading to alcohol-related organ injury in order to identify new targets to prevent or reverse disease progression.

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 (6). Although chronic alcohol consumption is not directly linked to the development of 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 people meeting the diagnostic criteria for alcohol use disorders (7, 8). 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% (7-9). The exact mechanisms by which alcohol mediates these effects on the lung are unknown, but experimental alcohol consumption has been associated with the up-regulation of pro-inflammatory cytokines (10-12), disruption of regulatory signaling pathways (13), activation of tissue remodeling (14, 15), and the induction of oxidative stress in rodent lungs (16). These factors contribute to the development of an "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) (6). Therefore, the primary goal of the current work is to investigate the mechanisms by which chronic alcohol sensitizes the lung to injury and inflammation.

Intraperitoneal (i.p.) injection of bacterial lipopolysaccharide (LPS), or endotoxemia-induced lung injury, is a common animal model employed to study

ALI and ARDS (17, 18). Indeed, this model has been demonstrated to cause sequestration of neutrophils in the pulmonary vasculature and interstitium (19, 20). 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 (21). Systemic administration of LPS (versus intratracheal) is a particularly relevant model to investigate mechanisms of alcohol-induced organ pathology. In fact, chronic alcohol consumption has been demonstrated to increase systemic LPS in patients (22), and this could in turn damage the lung. 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 (20). Others have demonstrated that chronic alcohol exposure enhances glutathione depletion and oxidative stress in a rat model of endotoxemia-induced ALI (16, 23). The effect of chronic alcohol exposure on endotoxemia-induced lung injury and inflammation in a mouse model has not been tested. The first goal of the current project is to characterize a mouse model of alcohol-enhanced acute lung injury.

A major focus of the Arteel group's recent research has been to investigate parallel and possibly interdependent mechanisms of liver and lung injury in the setting of chronic alcohol abuse. One proposed parallel mechanism of alcohol-induced liver and lung injury is the induction of tissue remodeling. The activation of the coagulation cascade is a key response to acute injury which transiently alters the extracellular matrix (ECM). Plasminogen activator inhibitor-1, or PAI-1, is a key

regulator of the coagulation cascade. PAI-1 is an acute phase protein normally expressed by adipocytes and endothelial cells, but is also expressed by other cells during times of inflammation and/or stress (24). This group has identified a 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 (25). Similar effects have also been demonstrated in other models of hepatic inflammation, including LPS-induced liver injury enhanced by partial hepatectomy (26) or ethanol pre-exposure (27). PAI-1 is also known to contribute to the development of ALI and ARDS (28, 29), and elevated PAI-1 in bronchoalveolar lavage (BAL) fluid in patients with these conditions is associated with increased mortality (30-32). PAI-1 induction has also been demonstrated to enhance susceptibility to LPS-induced ALI in animal models (33). PAI-1 induction also enhances ALI in a model of bleomycin-induced lung injury (34). Although the role of PAI-1 has been studied in many models of lung injury, including cancer (35), fibrosis (36), and ALI (37), the role of PAI-1 in alcohol-enhanced ALI remains largely unknown. Therefore, the second goal of the current work is to determine if PAI-1 is involved in alcohol-mediated pulmonary sensitization.

MATERIALS AND METHODS

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.

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 for further analysis. Prior to lavage of the lungs, 10 mL of 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 BAL fluid (BALF) were separated by centrifugation and removed from remaining BALF and fixed on slides for further analysis (38). Portions of lung tissue 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 (Tel-Test, Austin, TX) and chloroform: phenol separation (see RNA isolation Methods).

B. Chronic model of alcohol exposure

Eight week old male C57BL6/J and PAI-1 knockout (B6.129S2-Serpine1^{tm1Mlg}/J: PAI-1$^{-/-}$) mice were purchased from the Jackson Laboratory (Bar

Harbor, ME) and exposed to either ethanol-containing Lieber-DeCarli diet (Dyets, Inc.) 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 and 5 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-dextrin. 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, St. Louis, MO) or vehicle (saline). Animals were euthanized 4 or 24 h after LPS (or vehicle) injection.

Figure 1
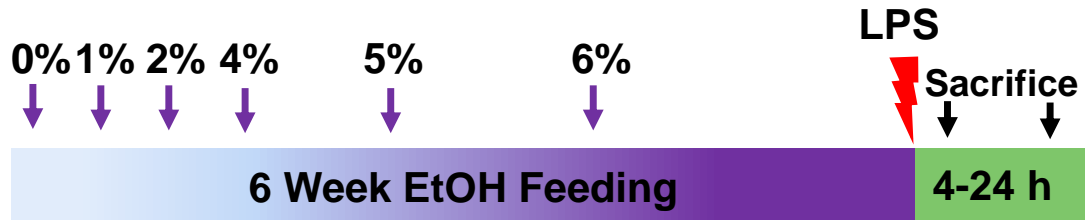
Figure 1: Lieber-DeCarli model of chronic alcohol exposure and experimental endotoxemia

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

C. Clinical chemistry

Plasma activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using standard kits (Thermotrace, Melbourne, Australia).

D. Histology

1. General morphology

Formalin fixed, paraffin embedded lung tissue was cut at 5 µm and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Fisher, 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 (Fisher, Waltham, MA).

2. Scoring of septal thickening and alveolar hemorrhage

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 (39). 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 (17). 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 (17).

Alveolar hemorrhage was scored using twenty blinded photographs per sample of hematoxylin and eosin-stained paraffin embedded lung tissues taken at 400x magnification. Each field was assessed for the presence of alveolar hemorrhage. Fields in which 0% of the tissue was hemorrhagic received a score of 0. Those with 1-25% hemorrhagic tissue received a score of 1, 26-50% received a score of 2, 51-75% received a score of 3, and 76-100% received a score of 4. The average score of 20 fields is reported.

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 µm and mounted on charged glass slides. Sections were deparaffinized with Citrisolv (Fisher, Waltham, MA) and rehydrated through graded ethanol. Tissue specimens were incubated in a solution of napthol AS-D chloroacetate (1 mg/ml) in N,N-dimethylformamide, with 4% sodium nitrite and 4% new fuchsin. The napthol AS-D chloroacetate is enzymatically hydrolyzed by chloroacetate esterase in neutrophils, liberating the napthol compound. Napthol 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

Immunohistochemical staining for markers of macrophages (F4/80) and oxidative stress (4-HNE) were performed as previously described (40). Briefly,

formalin-fixed, paraffin embedded sections (5 µm) were deparaffinized and rehydrated with graded ethanol solutions. Endogenous peroxidase activity was quenched by incubating slides in a 3% H_2O_2 solution for 5 minutes at room temperature.

For F4/80 staining, slides were incubated in an avidin-biotin blocking reagent (Dako, Carpinteria, CA) to reduce non-specific staining. Then, slides were incubated in primary antibody for the F4/80 cell surface receptor (1:50 in 10% goat serum; Abcam, Cambridge MA) for two hours at room temperature. The Vectastain Elite ABC kit was used for detection (Vector Laboratories, Inc., Burlingame, CA). Briefly, tissue was incubated in a biotinylated anti-Rat IgG secondary antibody for 15 minutes at room temperature. Tissue was then washed and incubated in a solution containing avidin-bound horseradish peroxidase (HRP) for 30 minutes.

4-HNE staining was detected using the UltraVision Detection System (Thermo Scientific, Fremont, CA). Briefly, after hydrogen peroxide blocking, slides were incubated in Ultra V Blocking Solution to reduce non-specific stain. Then, slides were incubated in primary antibody for 4-HNE (1:500 in PBST+1% BSA, Alpha Diagnostics, San Antonio TX) for 30 minutes at room temperature. For detection, slides were incubated in biotinylated goat anti-Rabbit IgG secondary antibody for 10 minutes at room temperature. Tissues were then washed and incubated in a solution containing avidin-bound horseradish peroxidase (HRP) for 10 minutes.

The HRP substrate 3, 3'-diaminobenzidine (DAB) was added to tissue sections until positive (brown) staining was visible macroscopically. Slides were

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counterstained with Hematoxylin (Dako, Carpinteria, CA) for 1 minute, washed, dehydrated through graded ethanol and then mounted with Permount (Fisher, Waltham, MA). Each slide contained a negative tissue section which does not receive primary antibody.

E. Myeloperoxidase Activity

Myeloperoxidase activity was measured as previously described (41, 42). 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.

F. 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, Ambion, Austin, TX). RNA concentrations were

determined spectrophotometrically and 1µg of total RNA was reverse transcribed using a kit (Quanta Biosciences, Gaithersburg MD).

The pulmonary mRNA expression of select genes was detected by quantitative reverse-transcription polymerase chain reaction (qRT-PCR), which is routine for this group (27). 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), MIP-2, KC, F4/80, MCP-1, MIP-1β, lungkine, and B-actin were purchased from Life Technologies. All primers were designed to cross introns and ensure that only cDNA and not genomic DNA was amplified. PerfeCta qPCR Fast Mix (Quanta Biosciences, Gaithersburg MD) was used to prepare the PCR mixture. This 2X mixture is optimized for TaqMan reactions and contains $MgCl_2$, dNTPs, and AccuFast Taq DNA Polymerase. Amplification reactions were carried out using the ABI StepOne Plus machine and software (Quanta Biosciences, Gatithersburg, MD) 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 (B-actin). This method determines the amount of target gene, normalized to an endogenous reference and relative to a calibrator ($2^{\Delta\Delta CT}$).

G. Statistical Analyses

Results are reported as means ± standard error (SEM; n = 3-6). ANOVA with Bonferroni's post-hoc test (for parametric data) or Mann-Whitney Rank Sum test (for non-parametric data) was used for the determination of statistical significance among treatment groups, as appropriate. A *p* value less than 0.05 was selected before the study as the level of significance.

RESULTS

Over the course of the six weeks of dietary exposure, ethanol-fed mice gained 3.2±0.4 g and pairfed mice gained 4.2±0.4 g. Ethanol feeding alone caused elevated plasma transaminase (ALT and AST), as did four hours of LPS exposure; however, these changes were not significant. Ethanol feeding significantly enhanced ALT levels 4 hours after LPS injection when compared with pairfed animals. AST levels were significantly elevated 24 hours after LPS injection in pairfed and ethanol-fed animals. These results are summarized in Table 1.

1. **Ethanol enhanced pulmonary damage caused by systemic administration of LPS.**

As expected, LPS administration caused pulmonary damage in the form of alveolar hemorrhage and thickening of the alveolar septa (21). Hemorrhage was seen as early as four hours after LPS as an increase in the number of red blood cells in the pulmonary intersitium (Figure 2, middle panels). Hemorrhage was macro-heterogeneous, with some areas of the pulmonary tissue experiencing more severe hemorrhage than others. Scoring of blinded photomicrographs (as described in Materials and Methods) revealed that alcohol exposure had no significant effect on the amount or severity of alveolar hemorrhage (Figure 3A). Significant thickening of the alveolar septa occurred four hours after LPS injection, as seen in H&E staining (Figure 2, middle

Table 1

	Body Weight (g)	Weight Gain (g)	EtOH Consumption (g/day)	ALT	AST
Pairfed	25.8±0.5	4.2±0.4	N/A	22±3	32±3
+ LPS (4h)				45±2	70±7
+ LPS (24h)				153±57	155±30[a]
EtOH	26.2±0.6	3.2±0.4	0.454±0.004	47±3	51±15
+ LPS (4h)				99±19[b]	95±16
+ LPS (24h)				110±33	172±26[a]

Table 1: Routine parameters and clinical chemistry

Data are presented as mean±SEM. [a], *p* < 0.05 compared to pairfed controls; [b], *p* < 0.05 compared to LPS alone

panels). Twenty-four hours after LPS injection, alveolar walls resembled that of control animals in pairfed animals (Figure 2, bottom left panel); conversely, alveolar walls in ethanol fed animals doubled in thickness at this time point (Figure 2, bottom left panel). Measurements of alveolar septa in blinded photomicrographs reveal that these differences are statistically significant (Figure 3B).

2. **Ethanol did not affect recruitment of neutrophils or macrophages to the lung after LPS injection.**

   Because ALI is associated with robust neutrophil infiltration into the pulmonary interstitium (43), the effects of alcohol on pulmonary neutrophil recruitment were assessed histologically and enzymatically. As expected, LPS injection caused a robust increase in the number of neutrophils in the pulmonary vasculature and interstitium, as indicated by an increase in the number of pink CAE-positive cells (Figure 4, left panel). This histological observation was associated with an increase in MPO activity (Figure 5A). Ethanol feeding did not significantly affect neutrophil recruitment compared to pairfed counterparts.

   Resident macrophages in many tissues, including in the lung, are often involved in inflammatory tissue damage. To determine if alcohol-enhanced pulmonary damage was caused by an increase in the number of alveolar macrophages, the number of alveolar macrophages was determined using immunohistochemical staining for the macrophage surface marker F4/80.

Figure 2
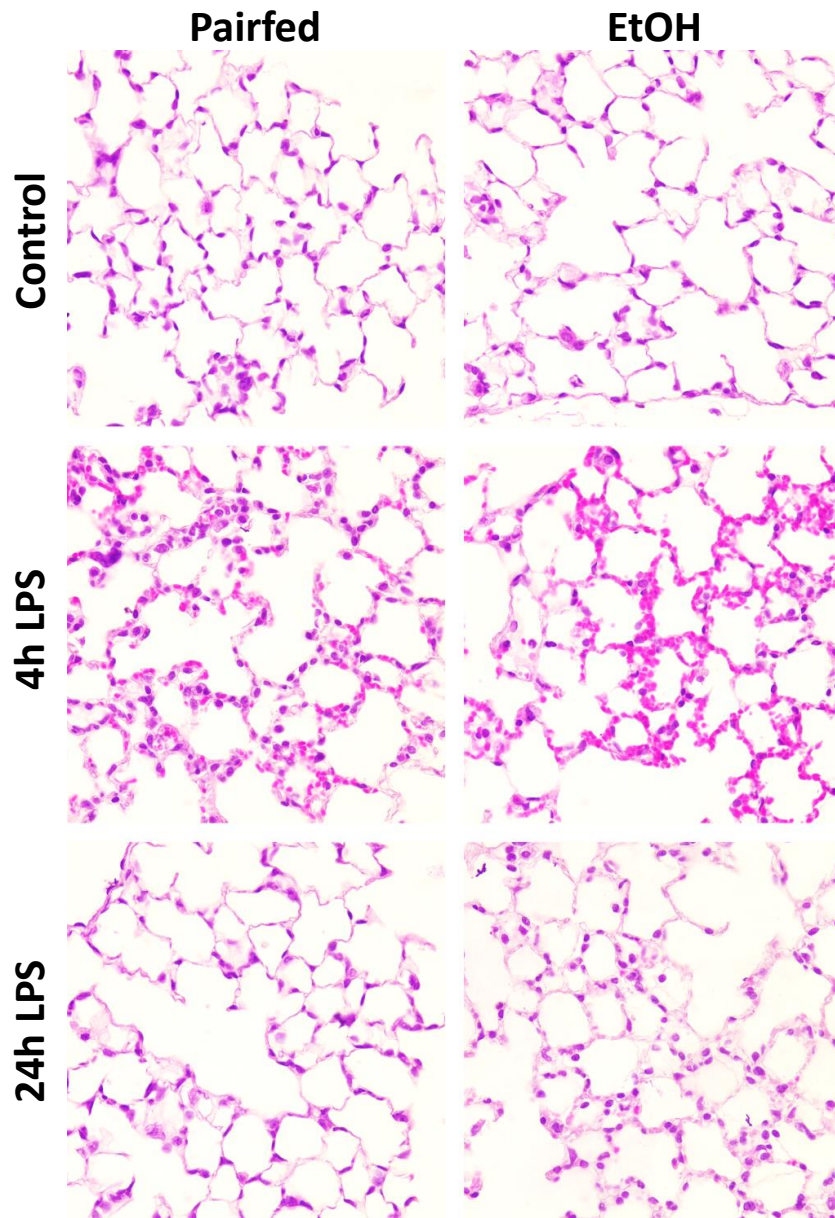
Figure 2: Effects of chronic alcohol on pulmonary injury after LPS injection.

Representative photomicrographs of lung tissue after hematoxylin and eosin staining (400x, H&E).

Figure 3
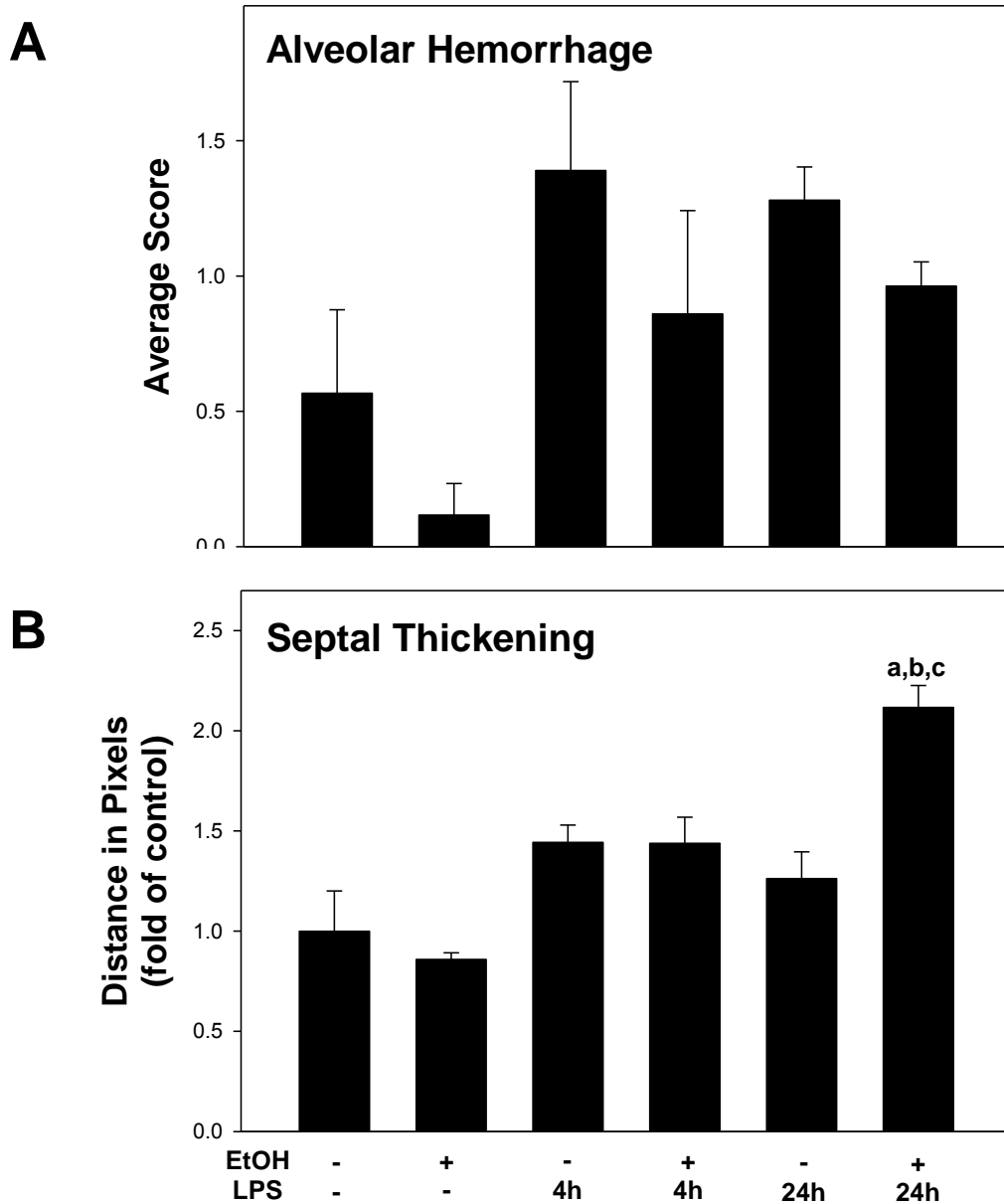
Figure 3: Effects of chronic alcohol feeding on indices of pulmonary damage.

Alveolar hemorrhage (A) was scored in blinded H&E-stained photomicrographs and septal thickness (B) was measured as described in Materials and Methods. Indices are reported as mean ± SEM. [a], $p < 0.05$ compared to pairfed controls; [b], $p < 0.05$ compared to LPS alone; [c], $p < 0.05$ compared to 4h LPS.

Histologically, LPS injection appeared to cause an increase in the number of F4/80-positive cells (Figure 4, right panel). Ethanol feeding did not have a significant effect on the number of F4/80 cells compared to LPS alone. This increase in F4/80-positive cells seen with LPS did not, however, correlate with an increase in F4/80 mRNA expression (Figure 5B).

3. **Alcohol exposure enhances expression of a subset of pro-inflammatory mediators.**

Acute lung injury is characterized by increased expression of several inflammatory mediators (17, 21). The effect of chronic alcohol feeding on the pulmonary inflammatory expression profile was determined using qRT-PCR (as described in Materials and Methods). Ethanol alone had no significant effect on expression of any inflammatory mediators measured, including TNFα, IL-6, IL-1β, or PAI-1 (Figure 6A). LPS injection significantly induced expression of these four mediators. Ethanol feeding significantly enhanced expression of the inflammatory cytokine IL-6. Expression of TNFα, IL-1β, and PAI-1 after LPS did not significantly change in animals exposed to chronic ethanol.

Because chemokines and subsequent neutrophil recruitment are known to play a key role in ALI (44), the expression of several chemokines were also determined (Figure 6B). Once again, ethanol feeding alone did not significantly alter expression of any of the chemokines measured, including MCP-1, MIP-1β, lungkine, MIP-2, and KC. LPS significantly induced expression of MCP-1, MIP-1β, MIP-2, and KC and significantly decreased lungkine expression. Alcohol feeding did not significantly affect expression of MCP-1, MIP-1β, or
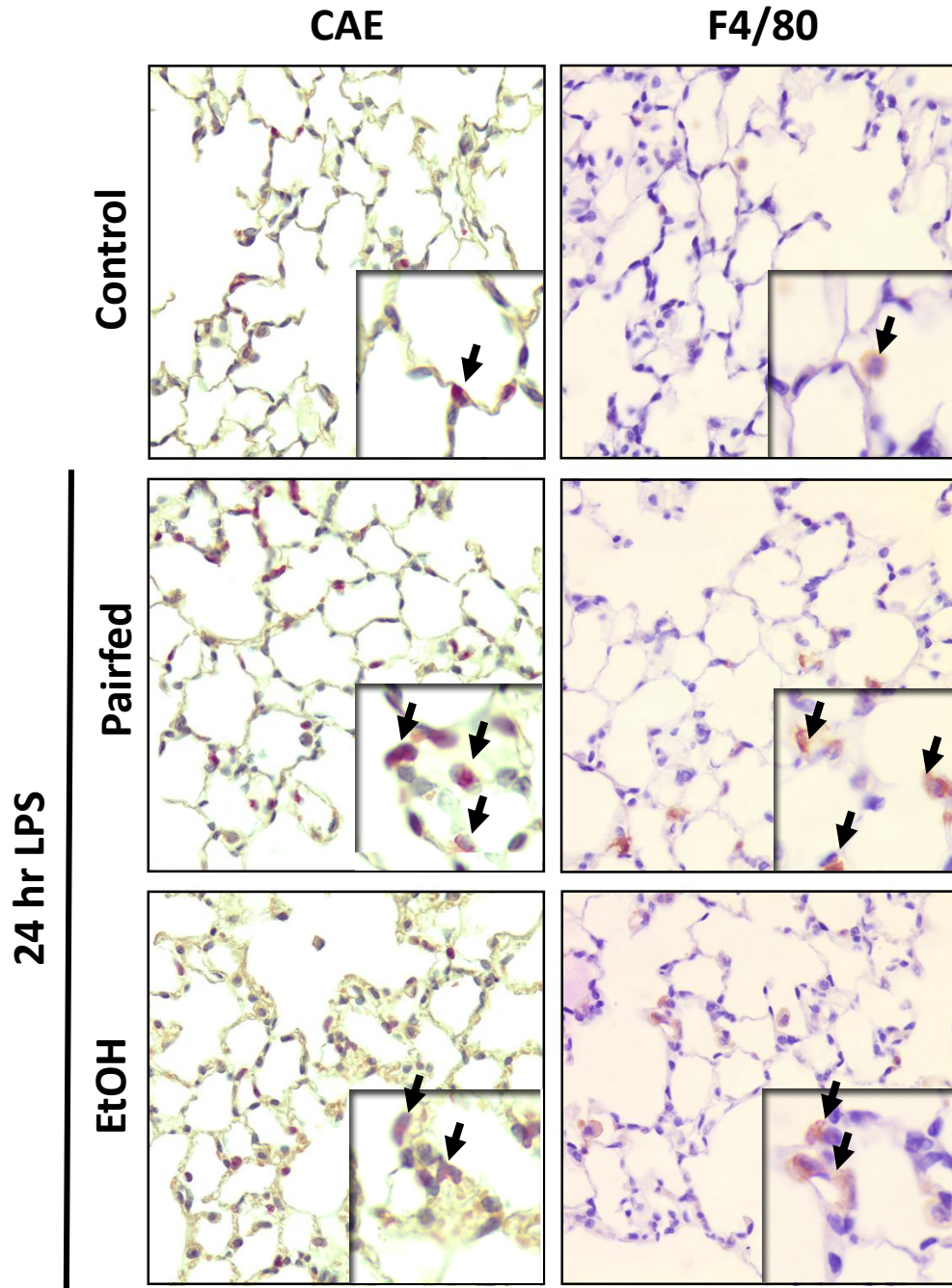
Figure 4

Figure 4: Effect of chronic alcohol feeding on pulmonary infiltration of neutrophils and macrophages.

Representative photomicrographs of paraffin-embedded lung tissue samples stained for CAE (left) and F4/80 (right) as described in Materials and Methods (400X). CAE- and F4/80-positive cells are indicated with arrows in insets.

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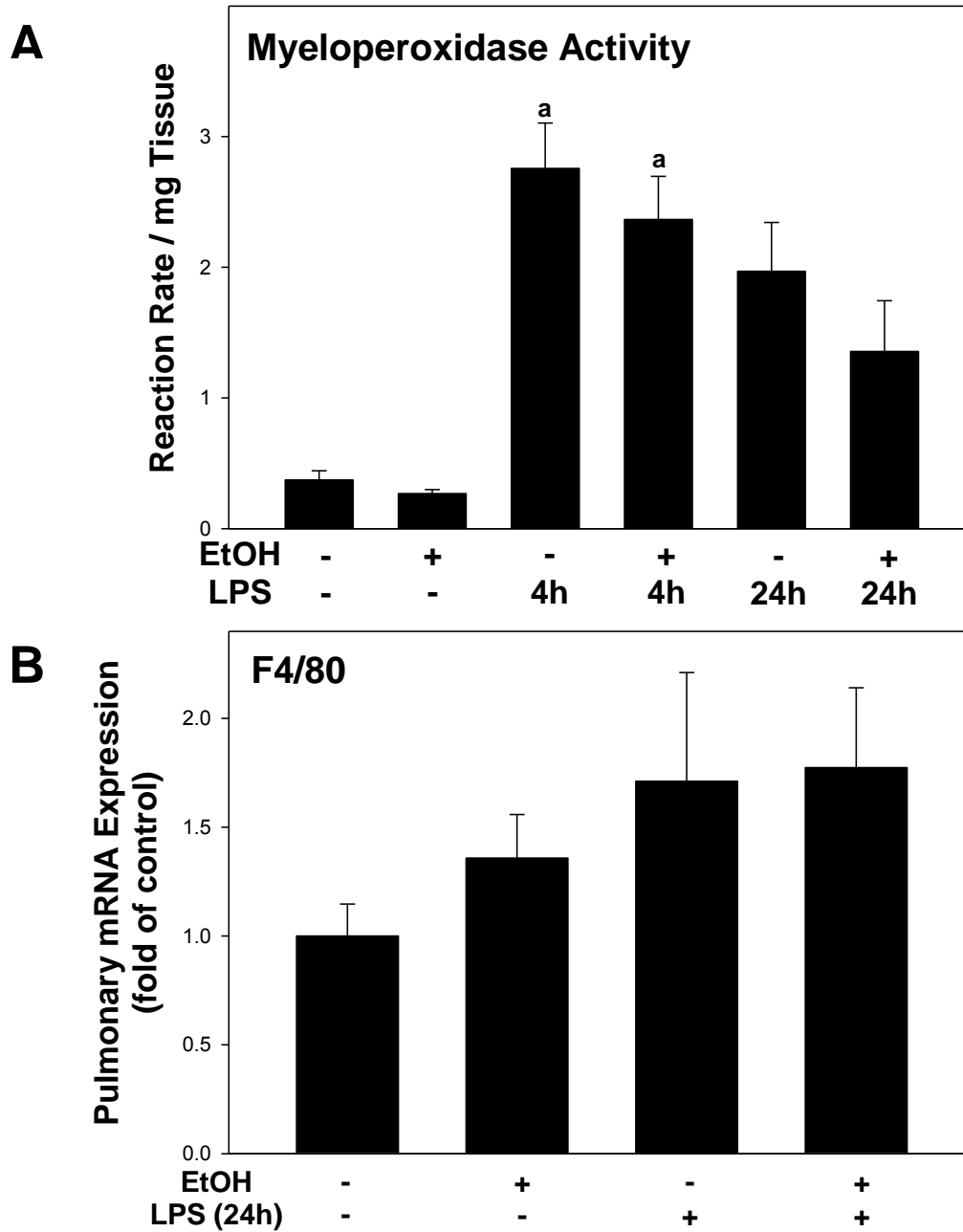
Figure 5

Figure 5: Effect of chronic alcohol feeding on myeloperoxidase (MPO) activity and F4/80 mRNA expression.

MPO activity (A) and F4/80 mRNA expression (B) were determined as described in Materials and Methods. Data are reported as mean ± SEM. [a], $p < 0.05$ compared to pairfed controls.

Lungkine after LPS. In contrast, alcohol feeding significantly enhanced expression of MIP-2 and KC after LPS.

Finally, expression of several adhesion molecules involved in leukocyte migration were measured, including ICAM-1, VCAM-1 and PECAM (Figure 6C). In a similar pattern to other inflammatory mediators measured, alcohol feeding alone had no significant effect on expression of adhesion molecules. LPS injection alone significantly increased expression of ICAM-1 and VCAM-1, but significantly decreased expression of PECAM. Ethanol feeding significantly enhanced expression of ICAM-1 and VCAM-1, but did not affect PECAM expression after LPS.

4. **Ethanol feeding enhanced oxidative stress after LPS injection.**

Others have shown that alcohol feeding causes glutathione depletion and oxidative stress in the lung (16). To determine the effect of chronic alcohol on oxidative stress in this model, lung tissues were stained immunohistochemically for the oxidative stress marker, 4-hydroxynonenol (4-HNE; Figure 7). As demonstrated by an increase in the amount of positive brown staining, ethanol feeding alone caused increased oxidative stress. LPS injection alone caused a similar increase in 4-HNE staining. Ethanol feeding increased both the amount and intensity of 4-HNE staining after LPS injection.

5. **PAI-1 deficient animals are protected against alcohol-enhanced lung injury.**

To determine the involvement of PAI-1 in this model of alcohol-enhanced acute lung injury, PAI-1 knockout mice were fed an ethanol-containing liquid

Figure 6
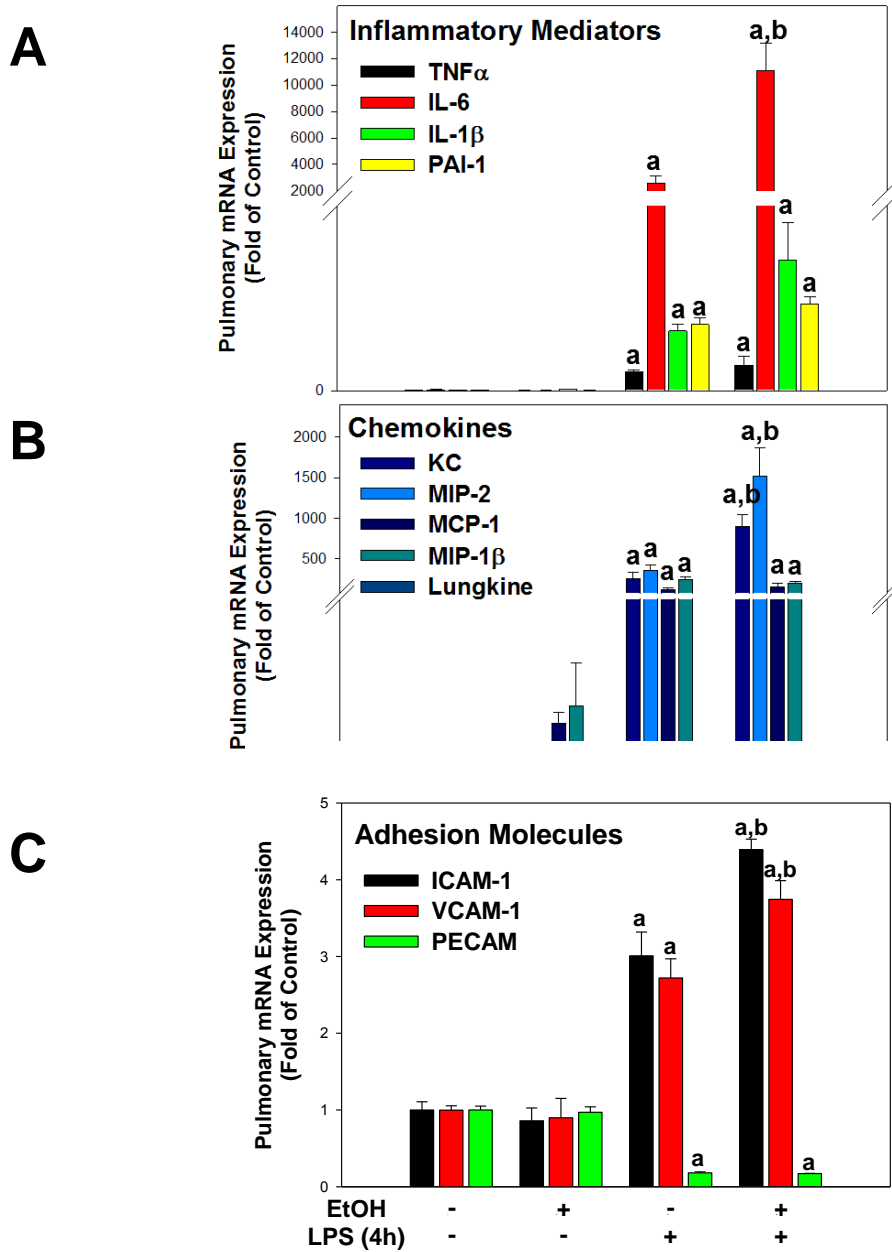
Figure 6: Effect of chronic alcohol feeding on LPS-induced expression of inflammatory mediators

The effects of alcohol feeding on expression of inflammatory mediators (A), chemokines (B), and adhesion molecules (C) were measured as described in Materials and Methods. Data are reported as mean ± SEM. [a], $p < 0.05$ compared to pairfed controls; [b], $p < 0.05$ compared to LPS alone.

Figure 7
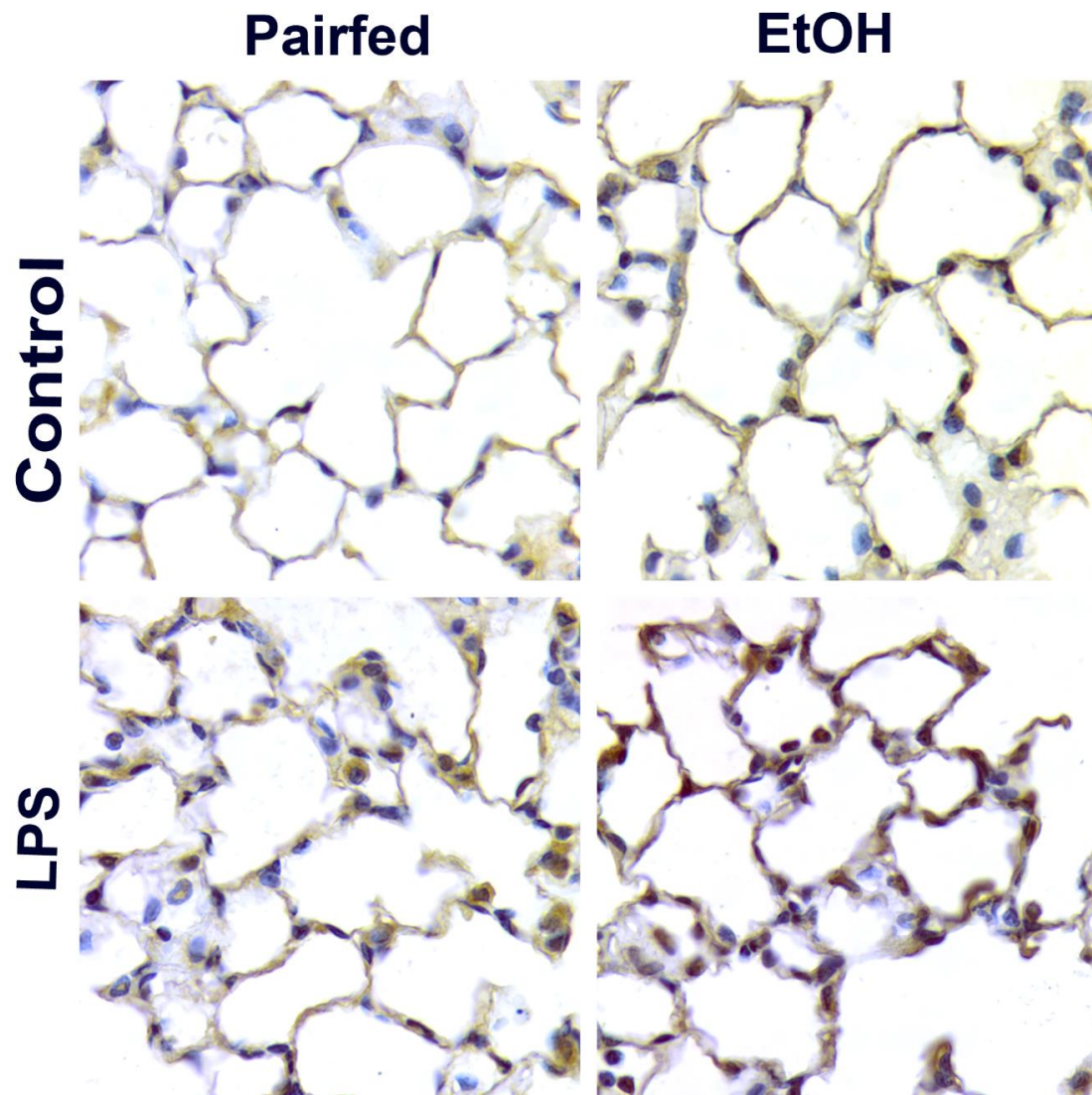
Figure 7: Effects of chronic alcohol feeding on oxidative stress after LPS injection (4 h).

Oxidative stress was assessed immunohistochemically in paraffin-embedded lung tissues using an antibody against the marker 4-HNE, as described in Methods and Materials. Representative photomicrographs are shown (400X).

diet for 6 weeks (as described in Materials and Methods). Ethanol-fed control PAI-1-/- animals showed no significant differences in alveolar septal thickness compared with ethanol-fed wild-type animals or pairfed wild-type animals. As previously demonstrated, chronic alcohol feeding cause significant septal thickening 24 hours after LPS administration. On the other hand, this septal thickening was significantly attenuated in PAI-1 knockout animals (Figures 8A and 8B). Additionally, as demonstrated previously, chronic alcohol feeding enhanced expression of the chemokines MIP-2 and KC, and the inflammatory cytokine IL-6. Enhanced IL-6 expression was blunted, albeit not significantly, in PAI-1 knockout animals. Ethanol-enhanced MIP-2 and KC expression were significantly attenuated in PAI-1 knockout animals. (Figure 8C).

Figure 8
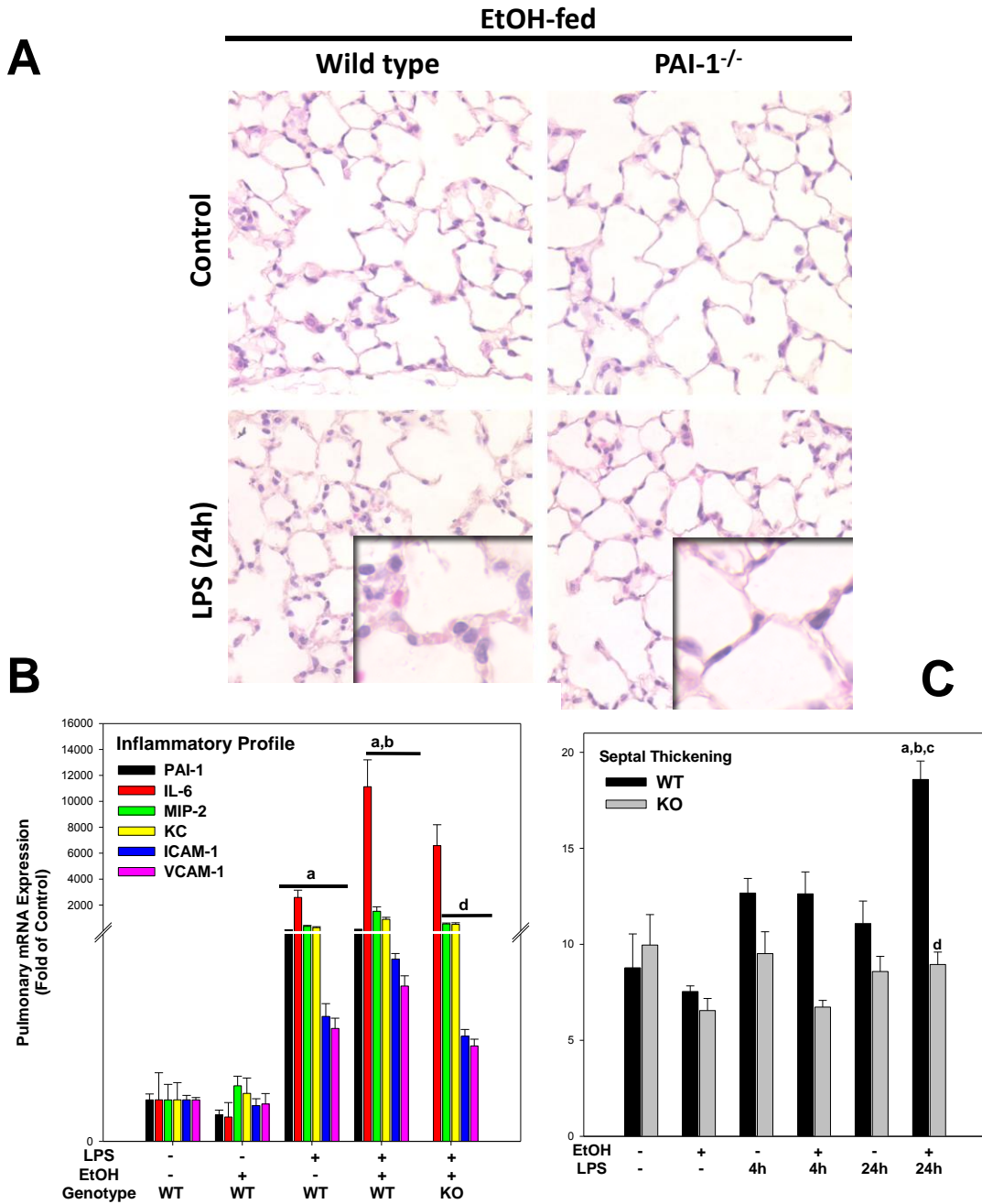
Figure 8: Effect of PAI-1 deletion on indices of alcohol-enhanced ALI

Representative photomicrographs of H&E-stained lung tissue sections (400X, A), expression of inflammatory mediators measured by qRT-PCR (B), quantification of septal thickening (as described in Materials and Methods) (C). [a], $p < 0.05$ compared to pairfed controls; [b], $p < 0.05$ compared to LPS alone; [c], $p < 0.05$ compared to 4h LPS; [d], $p < 0.05$ compared to WT animals

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DISCUSSION

Although chronic alcohol abuse does directly cause overt lung pathology, alcohol is known to increase susceptibility to severe lung injury, such as acute respiratory distress syndrome (ARDS) (7, 8).Therefore, the primary goal of this work was to characterize the effects of chronic alcohol exposure on inflammation and injury in a mouse model of acute lung injury in experimental endotoxemia utilizing systemically administered LPS. In accordance with previous studies (16, 21), experimental endotoxemia caused pulmonary injury, as evidenced by alveolar hemorrhage and septal thickening indicative of pulmonary edema. Chronic ethanol exposure did not affect the presence or severity of alveolar hemorrhage, but chronic ethanol did enhance and prolong septal thickening. These data are similar to findings by Holguin and colleagues, who determined that ethanol enhanced edematous lung injury in a rat model of ethanol pre-exposure and experimental endotoxemia (16). These data suggest that chronic alcohol exposure sensitizes the lung parenchyma to enhanced inflammatory damage.

In this model, chronic alcohol feeding had specific effects on the pulmonary inflammatory response after LPS. Alcohol alone did not cause a generalized increase in expression of pro-inflammatory mediators; instead, alcohol enhanced LPS-induced expression of a specific set of inflammatory mediators, including cytokines, chemokines, and adhesion molecules. One of these mediators was the

cytokine, IL-6. IL-6 is a pleotropic cytokine that is widely accepted to be involved in the acute phase response (45). Interestingly, IL-6 has been shown to have both pro- and anti-inflammatory properties (45). In fact, expression of adhesion molecules, including ICAM-1 and VCAM-1 has been demonstrated to be induced by IL-6 signaling, leading to enhanced neutrophil infiltration into target tissues (45, 46). Moreover, STAT3, which is activated by IL-6 has been shown to be critically involved in acute lung inflammation in a model of *Streptococcus* infection (47). Inhibition of STAT3 caused a dramatic decrease in M1-protein induced pulmonary edema, MPO activity, and CXC chemokine production.

On the other hand, IL-6 is an important cytokine in the resolution of inflammation. IL-6 is also known to be involved in the transition from the innate to the adaptive immune response, particularly the switch away from neutrophil recruitment to monocyte recruitment (48). Moreover, IL-6 drives differentiation of monocytes to macrophages. Others have shown that IL-6 knockout mice have enhanced pulmonary neutrophil recruitment, MIP-2 expression, TNF-α expression, and mortality in a model of LPS-induced acute lung injury (49). This thesis demonstrated that alcohol feeding drastically enhanced IL-6 expression after systemic LPS administration. This enhanced expression may be involved in increased neutrophil chemotaxis through the upregulation of the adhesion molecules ICAM and VCAM, as well as through the STAT3-mediated production of the CXC chemokines MIP-2 and KC. Conversely, enhanced IL-6 expression may be a compensatory mechanism involved in the resolution of alcohol-enhanced

inflammation. These opposing effects make IL-6 an interesting target for future study in this model of alcohol-enhanced ALI.

A hallmark of ARDS is a robust increase in the expression of the potent neutrophil chemoattractant, IL-8 (20). IL-8 has a well-defined role in ALI and ARDS through the recruitment and activation of neutrophils, and increased levels of IL-8 in the BALF of patients has been associated with an increased risk of developing ARDS, as well as increased mortality (44). In this model of alcohol-enhanced acute lung injury, chronic alcohol consumption enhanced expression of the murine IL-8 homologues, MIP-2 and KC. Although chronic alcohol robustly enhanced MIP-2 and KC expression caused by LPS, there was not an associated increase in recruitment of inflammatory cells, including macrophages and neutrophils. This suggests that while the number of neutrophils is unchanged, neutrophils in alcohol-fed animals may show increased activation, and therefore, increased tissue damage.

In fact, a marker of neutrophil activation is the generation of reactive oxygen species (ROS) for the killing and phagocytosis of pathogens- a phenomenon known as oxidative burst (50). Others have shown that IL-8 stimulation primes neutrophils for oxidative burst in a dose-dependent manner (51), suggesting that enhanced MIP-2 and KC expression (IL-8 homologues) may contribute to enhanced pulmonary damage due to neutrophil-mediated oxidative stress. Indeed, this work has demonstrated that chronic alcohol feeding causes enhanced oxidative stress after LPS administration. Additionally, others have demonstrated that oxidative stress itself can enhance IL-8 production (52). Previous work has

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demonstrated that alcohol administration causes oxidative stress in the lung via glutathione depletion (16). The work presented in this thesis has confirmed this finding in a murine model. These results suggest that alcohol, via the induction of oxidative stress, primes macrophages to enhanced chemokine production after an inflammatory stimulus, such as LPS. This enhanced IL-8 production (or MIP-2/KC in rodents) may enhance the oxidative burst of recruited neutrophils, leading to enhanced inflammatory damage of pulmonary tissues. This hypothesis is outlined in Figure 9.

Finally, the current work has identified a potential novel role of PAI-1 in alcohol-enhanced ALI. As mentioned previously, the Arteel group has identified a critical role of PAI-1 in the development of alcohol-induced liver injury and inflammation (26, 27). Indeed, PAI-1 knockout mice were protected against alcohol-induced liver injury, inflammation, and necrosis in an enteral model of chronic alcohol exposure (26). As mentioned previously, PAI-1 is also known to play a role in several models of experimental ALI (33, 34, 37). Interestingly, in the model of alcohol-enhanced ALI described here, PAI-1 expression was not significantly enhanced after LPS by alcohol feeding, yet PAI-1 knockout animals were protected against enhanced septal thickening and chemokine production. These results suggest that while PAI-1 itself may not be directly involved, it is involved in critical pathways in the development of alcohol-enhanced ALI. This makes PAI-1 a unique target for further mechanistic investigation.

Figure 9
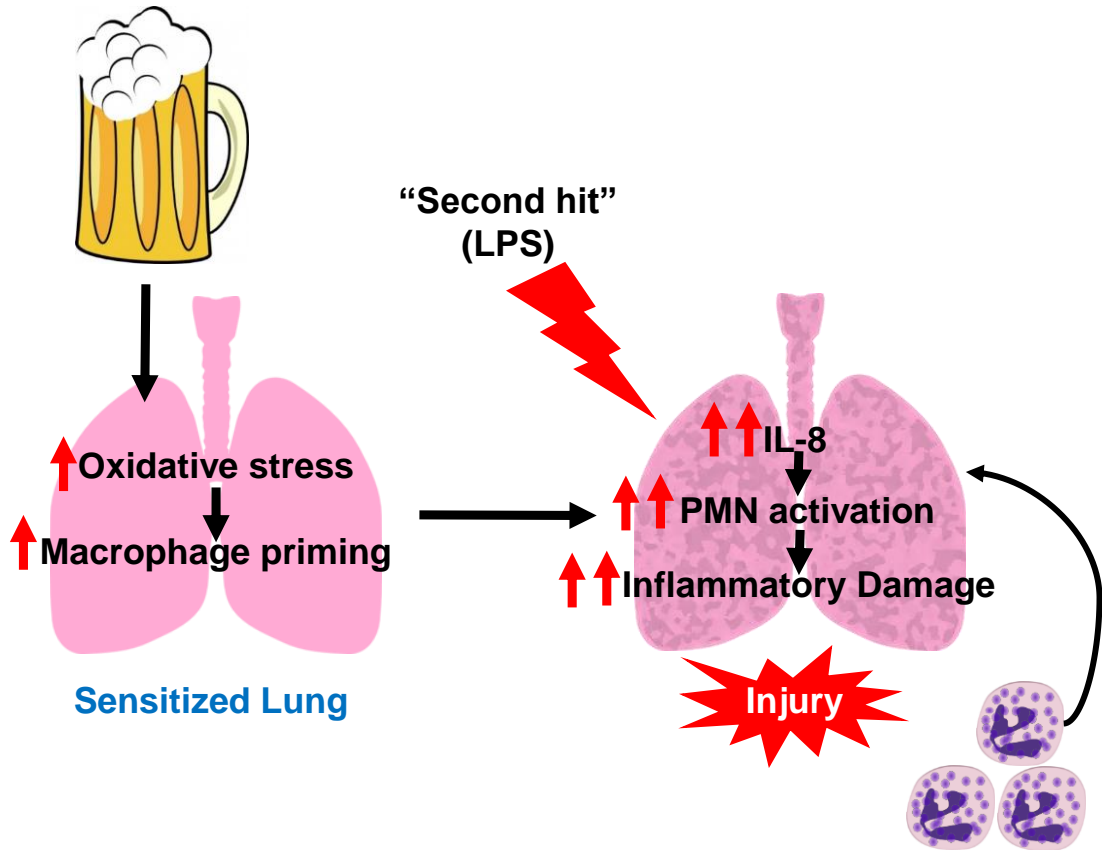
Figure 9: Proposed scheme of alcohol-enhanced pulmonary inflammation and injury

SUMMARY AND CONCLUSIONS

Strengths of this work

This thesis has many strengths. First, it establishes and characterizes a murine model of alcohol-enhanced ALI. Patients meeting the diagnostic criteria for an alcohol use disorder are 3.7 times more likely to develop acute respiratory distress syndrome (7, 8), suggesting that chronic alcohol sensitizes the lung to injury. The effects of chronic alcohol exposure on the development of lung injury have been investigated in other species (23), as well as in other models of alcohol administration (e.g. in drinking water). The characterization of the mouse model described in this work is particularly useful, however, because of the wide variety of genetically modified strains readily available at relatively low cost compared to other animal models.

Secondly, this work utilizes an *in-vivo* approach which accounts for the interaction of many pathways and organ systems. In particular, alcohol-induced organ pathology is complex, involving interactions between multiple organ systems, including the gut and the liver. Systemically administered LPS is a common model used to study ALI resulting from a systemic infection, and the model used for these studies is considered particularly relevant because of its ability to mimic sepsis-induced ALI (18). Intratracheal administration of LPS induces a robust inflammatory response, but confounding factors, such as aspiration injury, make it less applicable for the studies described in this thesis (20).

Additionally, these studies identified potential mechanisms by which alcohol sensitizes the lung to injury and reveal potential targets for therapy for alcoholic patients at risk for developing ARDS. Others have shown that alcohol administration causes pulmonary glutathione depletion and oxidative stress (16, 23, 53). It is also known that oxidative stress enhances expression of IL-8 from dendritic cells (52), which may then prime neutrophils to enhanced production of reactive oxygen species (ROS). Indeed, this work demonstrates that chronic alcohol exposure enhanced pulmonary oxidative stress after LPS administration. Furthermore, enhanced oxidative stress caused by ethanol pre-exposure was associated with enhanced alveolar septal thickening, a sign of tissue damage. Other studies have shown that N-acetylcysteine (NAC), a glutathione precursor, is protective in models of acute alcohol-induced liver injury (54). The lung is dependent on liver-derived glutathione (23), and alcohol intoxication depletes hepatic glutathione. Additionally, NAC has been shown to be protective in a model of LPS-induced ALI (55). Pretreatment with NAC has been shown to protect against hepatic glutathione depletion in mouse models (54), and may therefore protect the lung during alcohol-enhanced ALI. NAC has been used in clinical trials in alcoholic hepatitis in combination with other drugs with some effectiveness (56), and has improved intracellular glutathione pools and patient outcome in ARDS patients (57). Thus, it is possible that N-acetylcysteine could also protect the lung during alcohol-enhanced ALI and should be investigated as a potential therapy in future studies

Finally, this work has identified a novel role of PAI-1 in mediating the sensitizing effects of alcohol in the setting of acute lung injury. While PAI-1 is known to play a role in other models of acute lung injury (33, 34, 37), its role in alcohol-enhanced ALI has not been investigated. While the exact mechanism by which PAI-1 is involved in alcohol-enhanced ALI remains unclear, this study demonstrates that PAI-1 is critically involved in alcohol-enhanced chemokine production and inflammatory damage (e.g. alveolar septal thickening). These results introduce an interesting, novel target for future investigation.

Unanswered questions and future directions

<u>What is the role of PAI-1 in alcohol-enhanced ALI?</u>

The Arteel group has previously identified a critical role of PAI-1 in alcohol-induced liver injury and inflammation (26). Specifically, knocking out PAI-1 produced potent anti-inflammatory effects in a chronic enteral alcohol model (25). 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 involved in the coagulation cascade by degrading the extracellular matrix protein fibrin. Therefore, PAI-1 plays a major role in regulating fibrinolysis. The Arteel group has also demonstrated the critical role of PAI-1-induced fibrin accumulation in alcohol-induced liver injury (27). Ethanol pre-exposure enhanced LPS-induced fibrin deposition in the hepatic sinusoidal space, and inhibiting fibrin deposition protected against enhanced liver injury and inflammation.

The work described in this thesis has identified PAI-1 as a key mediator in alcohol-enhanced lung injury and chemokine production, despite the fact that PAI-1 induction is not significantly enhanced by alcohol feeding. Future experiments should determine the role of PAI-1 in alcohol-enhanced ALI, particularly the role of PAI-1-mediated fibrin accumulation.

<u>What is the role of ECM remodeling in alcohol-enhanced ALI?</u>

One mechanism by which PAI-1 may contribute to alcohol-enhanced ALI is by mediating ECM remodeling. In addition to its role in fibrinolysis, PAI-1 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 $\alpha_V\beta_3$. Previous work from 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 integrin $\alpha_V\beta_3$ signaling is critical for the progression of liver injury. Alveolar macrophages bind with high affinity to fibronectin and vitronectin ECM via the integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$, respectively. Chronic alcohol exposure has been shown to cause deposition of a fibronectin-enriched pulmonary ECM. PAI-1 induction can inhibit vitronectin-integrin interactions, impairing tissue repair (66). Thus, changing the ECM substratum can alter integrin signaling, and subsequently

the phenotype of the surrounding cells. For example, ligation of the integrin $\alpha_v\beta_3$ has been shown to enhance expression of pro-inflammatory cytokines via NFκB activation (67), and $\alpha_5\beta_1$-mediated macrophage binding to fibronectin can also be pro-inflammatory (62). Future research will examine the role of PAI-1-mediated ECM alterations, and how this altered ECM profile may affect integrin signaling, and therefore, the inflammatory response.

Is the liver involved in alcohol-enhanced lung injury?

A recent focus of the Arteel group has been to investigate parallel, and potentially interdependent mechanisms of liver and lung injury in the setting of chronic alcohol exposure. For example, the current work has identified PAI-1 activity as a potential parallel mechanism of organ injury. Additionally, the work presented here, and additional work from the Arteel group suggests that liver-derived mediators (e.g. TNFα (68)) may be directly involved in alcohol-enhanced lung inflammation. For example, the Arteel group has recently found that administration of the TNFα-blocking drug, etanercept, prevented alcohol-enhanced induction of MIP-2 and KC after LPS administration (Massey et. al., *under review*). Additionally, Siore and collaborators demonstrated a possible interaction between the liver and lung in an *in situ* perfused piglet preparation (69). This model revealed that hypoxemia and pulmonary edema were only observed when the liver and lung circulations were connected. Their results indicate that while endotoxemia could directly cause pulmonary vasoconstriction and leukocyte sequestration, interaction between the liver and lung was required for severe

inflammatory response and oxidative injury to the lung. Future research should elucidate and solidify this potential link between the liver and the lung.

Lastly, PAI-1 is a known downstream target of TNFα signaling. Future research should investigate the role of hepatic-derived TNFα in the induction and activity of PAI-1 in this model of alcohol-enhanced ALI.

Conclusions

In conclusion, this thesis has established and characterized a mouse model of alcohol-enhanced acute lung injury. It was determined that alcohol enhances LPS-induced alveolar septal thickening, as well as LPS-induced pulmonary expression of IL-6 and the murine IL-8 homologues MIP-2 and KC. These effects were potentially mediated by oxidative stress. This thesis also identified PAI-1 as a novel mediator of alcohol-enhanced pulmonary injury. This thesis is of interest because it establishes a mouse model which can be used in future studies to elucidate the mechanisms by which alcohol consumption sensitizes the lung to further injury. Although this thesis has characterized many aspects of ALI, ALI is a complicated disease state, and no single animal model is able to recapitulate all clinical aspects of the disease. However, this model has proven useful for the study of edematous injury and cytokine/chemokine production – two highly relevant clinical markers of ARDS. This model will be useful in future studies, including investigation of the role of PAI-1 and PAI-1-mediated ECM remodeling in alcohol-enhanced pulmonary injury.

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ABBREVIATIONS

4-HNE	4-hydroxynonenol
ALI	Acute lung injury
ALT	Alanine transaminase
ARDS	Acute respiratory distress syndrome
AST	Aspartate transaminase
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
CAE	Chloroacetate esterase
DAB	3, 3'-diaminobenzidine
EtOH	Ethanol
H&E	Hematoxylin and eosin
HTAB	Hexadecyltrimethylammonium bromide
ICAM-1	Intracellular adhesion molecule 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
I.P.	Intraperitoneal
KC	Keratinocyte-derived chemokine
KO	Knockout
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein 1
MIP-1β	Macrophage inflammatory protein 1 beta

MIP-2   Macrophage inflammatory protein 2

MPO   Myeloperoxidase

PAI-1   Plasminogen activator inhibitor one

PECAM   Platelet endothelial cell adhesion molecule

qRT-PCR   Quantitative reverse-transcription polymerase chain reaction

ROS   Reactive oxygen species

STAT3   Signal transducers and activators of transcription 3

TNFα   Tumor necrosis factor alpha

VCAM-1   Vascular cell adhesion molecule 1

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HONORS AND AWARDS

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

2015	Judge, Our Lady of Lourdes Sixth and Seventh Grade Science Fair
2012	Mentor, The College Prep Program, Western Middle School
2012	Administration, La Casa Latina

EDUCATION ACTIVITIES

Lectures/labs

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

Professional Development

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

LEADERSHIP

2013-2015   Class Representative, Department of Pharmacology and
              Toxicology, University of Louisville

2010-2012   President, University of Louisville Polo Club

FUNDING

2013-2015   Graduate Research Fellowship, Integrated Programs in
              Biomedical Sciences

ABSTRACTS AND PRESENTATIONS

ORAL PRESENTATIONS

Local/Regional

1. Oral Presentation, 06/15 Ohio Valley Society of Toxicology Summer
   Student and Postdoc Meeting, Cincinnati, OH
2. Research Seminar, 05/15 University of Louisville, University of Louisville
   Alcohol Research Center, Louisville, KY.
3. Research Seminar, 03/14 University of Louisville, Seminar in
   Pharmacology and Toxicology, Louisville, KY.

POSTERS

Local/Regional

1. **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
2. **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.
   Research!Louisville, Louisville KY
3. **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
4. **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

ABSTRACTS

National

1. 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
2. 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.
3. 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**, 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

2. **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

3. 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

4. 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

5. **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

6. Massey VL, **Poole LG**, Schmidt RH, Arteel GE (2012) Integrin $\beta3^{-/-}$ mice are protected against enhanced alcohol-induced liver injury. Research!Louisville Louisville, KY

7. **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. 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 (in preparation)
2. Massey VL, **Poole LG** Torres E, Schmidt RH, Ritzenthaler J, Roman J, Arteel GE Characterization of a model of concomitant liver and lung injury in a 2-hit model of chronic alcohol exposure (under review)