The Gut-Liver Axis Regulates Pulmonary Inflammation After Intoxication and Burn Injury

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LOYOLA UNIVERSITY CHICAGO

THE GUT-LIVER AXIS REGULATES PULMONARY INFLAMMATION AFTER INTOXICATION AND BURN INJURY

A DISSERTATION SUBMITTED TO
THE FACULTY OF THE GRADUATE SCHOOL
IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

PROGRAM IN INTEGRATIVE CELL BIOLOGY

BY

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CHICAGO, ILLINOIS

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ACKNOWLEDGEMENTS

I would like to thank my family for their love and my fellow lab mates for making me look forward to work each day. Together with my mentor, Liz, they have made this part of my training a truly memorable and enjoyable experience. There are not enough pages in this document that would be needed to express my gratitude to Liz for her selfless mentorship, coffee, food, life advice, and support. I will remember and cherish her guidance and friendship for many years to come. Finally, I would also like to thank my committee for investing in my training and education as well as acknowledge the National Institutes of Health, the American Medical Association Foundation, and the Stritch School of Medicine MD/PhD program for funding.
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<td>Abx</td>
<td>antibiotics</td>
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<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>$\alpha_{2A}$-AR</td>
<td>alpha-2a adrenoceptor</td>
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<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
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<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>activator protein-1</td>
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<tr>
<td>APR</td>
<td>acute phase responses</td>
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<td>ARDS</td>
<td>acute respiratory distress syndrome</td>
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<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
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<tr>
<td>BAC</td>
<td>blood alcohol content</td>
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<tr>
<td>BUN</td>
<td>blood urea nitrogen</td>
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<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>CFU</td>
<td>colony forming unit</td>
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<tr>
<td>CLO</td>
<td>clodronate</td>
</tr>
<tr>
<td>CoA</td>
<td>coenzyme A</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
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ICAM-1  intracellular adhesion molecule-1
IkB       inhibitor of kappa B
IKK       IkB kinase
IL        interleukin
i.p.      intraperitoneal
IRAK      interleukin-1 receptor-associated kinase
LBP       LPS binding protein
LPS       lipopolysaccharide
LW:BW     liver weight to total body weight ratio
MAPK      mitogen-activated protein kinase
MLCK      myosin light chain kinase
MLN       mesenteric lymph node
NAD       nicotinamide adenine dinucleotide
NADPH     nicotinamide adenine dinucleotide phosphate
NF-κB     nuclear factor kappa B
OCT       optimal cutting temperature
P         phosphorylated
PMN       neutrophil
p38i      p38 inhibitor SB203580
PIK       permeant inhibitor of MLCK
qRT-PCR   quantitative real-time polymerase chain reaction
ROS       reactive oxygen species
<table>
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<tr>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>SIRS</td>
<td>Systemic Inflammatory Response Syndrome</td>
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<td>TBSA</td>
<td>total body surface area</td>
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<tr>
<td>TG</td>
<td>triglyceride</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>TBSA</td>
<td>total body surface area</td>
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<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
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<td>ZO-1</td>
<td>zonula occludens</td>
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CHAPTER 1

INTRODUCTION

The widespread and rapidly increasing trend of binge drinking is accompanied by a concomitant rise in the prevalence of trauma patients under the influence of alcohol at the time of their injury. Epidemiologic evidence suggests up to half of all adult burn patients are intoxicated at the time of admission and the presence of alcohol is an independent risk factor for death in the early stages post burn. The major site of alcohol metabolism and toxicity, the liver is crucial to post burn outcome and experimental evidence implies an injury threshold exists beyond which burn-induced hepatic derangement is observed. Alcohol may lower this threshold for post burn hepatic damage through a variety of mechanisms including modulation of extrahepatic events, alteration of the gut-liver axis, and changes in signaling pathways. The direct and indirect effects of alcohol may prime the liver for the second-hit of many overlapping physiologic responses to burn injury. In an effort to gain a deeper understanding of how alcohol potentiates post burn hepatic damage we proposed studies to identify the mechanism of how ethanol intoxication prior to burn injury drives the aberrant inflammatory response relative to either ethanol or burn alone.

The resident macrophages in the liver, known as Kupffer cells, coordinate the hepatic response after injury and are a producer of systemic cytokines such as IL-6. Cytokine production by Kupffer cells can be induced by gut-derived lipopolysaccharide
(LPS), which can be found in the portal system after the combined injury due to intestinal barrier breakdown. Furthermore, ethanol can sensitize Kupffer cells to LPS through downstream signaling proteins such as mitogen-activated protein kinases (MAPK) leading to even greater IL-6 production after encountering LPS. This is important, as we have previously demonstrated that increased IL-6 after this combined injury drives pulmonary inflammation. With this knowledge, we hypothesize that binge ethanol intoxication exacerbates the hepatic response to burn injury through a feed-forward loop involving increased intestinal permeability, ethanol-induced sensitization of Kupffer cells to LPS via MAPK signaling, and hepatic IL-6 production. To test this hypothesis, three aims are proposed: 1) to determine the effects of alcohol intoxication on the post burn hepatic response, 2) if restoring gut barrier function or reducing intestinal microbial burden attenuates hepatic damage and IL-6 production, and 3) identify the role of Kupffer cells and any specific MAPK isoforms responsible for LPS-induced production of IL-6. Furthermore, the effects on pulmonary inflammation will be assessed for each aim.

Hepatic damage will be evaluated through serum markers and histologic analysis. Cytokine production will be measured at the mRNA and protein levels. We will limit bacterial translocation by restoring intestinal barrier function after injury with permeant inhibitor of kinase (PIK) and via prophylactic sterilization of the gut with oral antibiotics. The role of Kupffer cells will be determined by selectively depleting them before injury with clodronate liposomes. Kupffer cells will be isolated and assessed for MAPK phosphorylation and IL-6 production. Finally, MAPK isoforms identified as important in the Kupffer cell response to the combined injury will be inhibited \textit{in vivo}. These studies
will help gain an understanding of the mechanisms by which alcohol worsens post burn outcome and are aimed at identifying novel therapeutic targets in the clinically relevant setting of burn injury preceded by ethanol intoxication. As ethanol is a risk factor for many types of injury, a benefit may also apply to other forms of trauma.
CHAPTER 2

REVIEW OF RELATED LITERATURE:
ALCOHOL MODULATION OF THE POST BURN HEPATIC RESPONSE

Socioeconomic and Clinical Consequences

Alcohol is the most abused substance in the US and the third leading cause of preventable death [1], much of which is associated with unintentional injury [2,3]. Binge drinking, defined as consuming five or more drinks on one occasion, is an increasingly prevalent form of intoxication [4] and is the characteristic pattern of drinking among patients presenting with traumatic injury, including burns [5]. Nearly 50% of patients admitted for burns have a positive blood alcohol content (BAC) and these patients have worse clinical outcomes than individuals who sustain similar injuries not under the influence [6,7,8]. Specifically, they are twice as likely to acquire an infection, require more surgical procedures and have a longer duration of stay in the ICU than their nonintoxicated counterparts [6,8,9]. As a result of these complications, burn patients with prior alcohol exposure have been identified as a patient population at high risk for death in the early stages post burn [10]. With 1 million burn injuries in the US each year, resulting in 40,000 hospitalizations [11], this represents a substantial burden of morbidity, mortality and socioeconomic cost that is worsened by alcohol abuse. Despite the established consequences, there are currently few differences in the treatment and management of burn patients with and without prior alcohol exposure. The increased
morbidity exhibited by patients with pre-injury alcohol exposure may be due to an early increased inflammatory response and subsequent immune suppression [12,13], though the inciting cells and mechanisms responsible for these phenomena remain largely unknown.

As a responsive organ with metabolic, hormonal and immune functions, the liver is central to the physiologic response to burn injury and plays a pivotal role in post burn outcome [14]. This is highlighted by the close correlation between hepatic function and post burn prognosis as seen in a national sample of 31,338 adult burn patients [15]. Correspondingly, hepatic cirrhosis, often caused by chronic alcoholism, is a high premorbid contributor to death in the setting of severe burn (3). Given the comorbidities associated with chronic alcoholism, it is not surprising that clinical and animal studies demonstrate amplified liver damage when chronic alcohol exposure precedes thermal injury. In recent years, however, the prevalence of binge drinking has increased precipitously and now represents the majority of alcohol consumed in the US [4]. Due to its high association with injury in an otherwise healthy population, binge drinking may be the more clinically relevant mode of alcohol abuse in burn patients today [5,16]. Indeed, more recent studies suggest the majority of intoxicated burn patients are now binge drinkers not chronic alcoholics [6]. Animal studies also confirm using various doses and timing before injury that the presence of intoxication at the time of injury, even at a single dose, is the key factor in the ability of alcohol to worsen post burn outcome. Therefore we focus this review on the potential mechanisms of how alcohol, consumed acutely as a binge, affects the post burn hepatic response.
The total body surface area (TBSA) affected by a burn is a major factor in determining the severity of injury, with increased TBSA correlating to increased systemic inflammation [17]. In an animal model, substantial hepatic derangements were present after a 40% TBSA burn that were absent when reduced to 20% TBSA [18]. This may suggest an injury threshold beyond which an aberrant hepatic response occurs. Work from our lab and others demonstrate significant hepatic damage in a mouse model of 15-20% TBSA burn only when intoxication precedes the injury [19,20,21,22,23]. This may indicate that alcohol lowers the threshold for an impaired post burn hepatic response. Indeed, alcohol exposure and burn injury have many overlapping physiologic effects that work in a synergistic manner to cause hepatic derangement. While burn-induced liver dysfunction may last for years after the initial injury [24], we center this review on the short term response in hopes of better characterizing the early events that may predispose to the later hypermetabolic state. We summarize below potential mechanisms of alcohol’s ability to worsen post burn outcome through extrahepatic events, direct effects on the liver and alterations in signaling pathways before discussing existing treatment regimens.

**Extrahepatic Events**

**Intestinal Ischemia**

Animal studies demonstrate catecholamine-mediated vasoconstriction of the splanchnic vasculature makes the intestines particularly susceptible to ischemic injury after burn [25]. Decreased cardiac output in the early phases post burn [26] worsens circulating blood flow, leading to intestinal damage and a loss of barrier function [27,28]
in rodents. A single intoxication event with alcohol prior to a burn injury in mice enhances intestinal damage leading to greater dissociation of tight junction complexes in the intestinal epithelium [29,30,31] and resultant permeability [32,33] compared to either insult alone. The mechanism for alcohol’s ability to exacerbate post burn intestinal damage may stem from its action on the post burn hemodynamic response and tissue oxygen requirements. We recently reported that alcohol given as a single oral gavage (1.1 g/kg) potentiates post burn bradykinin signaling in mice, leading to greater extravasation of fluid from the vascular compartment compared to a burn alone [34]. This adds a degree of hypovolemia into a scenario in which organs supplied by the splanchnic vasculature are already at risk for ischemic injury [28]. Likewise, clinical data demonstrate intoxicated burn patients require more aggressive fluid resuscitation than do their non-intoxicated counterparts [6], also indicating a shift in fluid compartments. Choudhry et al. demonstrated that a BAC of 100 mg/dl before burn injury in rats decreases blood flow and oxygen delivery to the intestines and liver [35]. Additionally, animal studies indicate that within 90 minutes of drinking, a single dose of alcohol increases oxygen demand in the liver by 48% [36] with oxygen consumption rate doubling in the liver after 2.5 hours of alcohol exposure [37]. While this does not rule out the influence of other effects of alcohol in post burn remote organ ischemia, it points towards the ability of alcohol to enhance post burn third spacing of fluid while creating a hypermetabolic state, widening the disparity between oxygen delivery and requirement in the intestines and liver.
The high baseline bacterial content of the intestines represents an enormous potential reservoir for systemic infections and sepsis after injury [38,39,40]. Trauma, such as a burn, causes a disruption in normal peristalsis leading to bacterial overgrowth in the intestine and this bacterial load is even greater when alcohol ingestion precedes the injury in rats [33]. While the exact mechanism by which alcohol enhances post burn bacterial growth in the intestine remains unknown, experimental evidence points to modulation of hormonal control [41], shifts in intestinal flora population [42], and the promotion of adynamic ileus [43] as possible causes, as reviewed elsewhere [44]. Animal models report that after disruption of intestinal barrier integrity, bacteria and bacterial products such as lipopolysaccharide (LPS) translocate from the lumen of the intestine into the lymphatic [45,46] and portal [47] systems. The number of bacteria found in the mesenteric lymph nodes is directly proportional to the degree of increased bacterial growth in the intestines after injury [33], suggesting important roles for both the luminal bacterial burden and the degree of intestinal permeability. As both bioload and permeability are exacerbated with the combined injury, it is befitting that many animal studies report up to 10 times greater amounts of bacterial translocation when alcohol precedes a burn than after a burn alone [19,29,30,32,34,48]. Much of the translocated bacteria and LPS will pass through the liver, which receives 75% of its blood supply from the portal system [14] and is therefore sensitive to portal system hemodynamic changes and content. As the largest reticular endothelial system organ and the first to encounter
portal blood, the liver plays an important role in clearing live intestinal bacteria and LPS. The relationship between intestinal microbiota and the liver is known as the “gut-liver axis” and is proposed to regulate a myriad of human diseases, such as nonalcoholic fatty liver disease, primary sclerosing cholangitis, celiac disease, and others [49,50,51,52,53]. This interaction is prominent after burn injury as seen in autopsies of burn patients where the amount of bacterial translocation correlates with the degree of steatosis [54]. We have previously reported that in mice, antecedent ethanol exposure (single dose of 1.1 g/kg) potentiates post-burn intestinal damage [19,29,48], hepatic steatosis [23,55] and IL-6 production [19,21,48,56] compared to either intoxication or a burn alone. Furthermore, limiting crosstalk between the gut and liver after injury, either through restoration of tight junction complexes or prophylactic sterilization of the gut, attenuated hepatic derangements to the level of a burn alone [20]. This suggests that the increased hepatic damage seen when intoxication precedes a burn stems from the ability of alcohol to alter the gut-liver axis (Figure 1). In addition to increasing the delivery of LPS into the portal system after burn, alcohol also modifies the post burn hepatic response through its influence on Kupffer cells in the liver.
Figure 1. The gut-liver axis after burn injury. Burn injury leads to intestinal damage, allowing LPS to enter the portal system and illicit a hepatic response which can contribute to pulmonary inflammation.

**Intrahepatic Mechanisms**

**Kupffer Cell Sensitization to LPS**

Representing the body’s largest tissue-fixed macrophage population, Kupffer cells continuously sample portal blood for foreign antigen and orchestrate the hepatic response to injury [57]. Kupffer cells can become activated when LPS binds to Toll-like receptor 4 (TLR4), an interaction dependent on its co-receptor, CD14 [58] and enhanced in the presence of LPS binding protein (LBP) [59]. TLR4 signaling can result in a variety of downstream signaling pathways, many of which result in pro-inflammatory cytokine production [60,61]. At low levels, cytokines, such as interleukin-6 (IL-6), are beneficial to hepatocyte survival, but exposure to levels above an acceptable threshold or for a prolonged duration can be detrimental, as seen in a mouse model [62]. Kupffer cells protect themselves from overstimulation by expressing low levels of CD14 relative to
other monocytes [63]. After burn injury, Kupffer cells fulfil the role of clearing gut-derived LPS from the portal system while avoiding over-stimulation. The importance of maintaining homeostatic Kupffer cell function in the injury setting was nicely demonstrated in a model of systemic inflammation where Kupffer cell depletion increased gut-origin septicemia but attenuated toxic symptoms associated with macrophage hyperactivity [64]. Animals studies have shown that hyperactive Kupffer cells can cause hepatic damage after many types of injury, including hemorrhagic pancreatitis [65], trauma and sepsis [66], as well as burns [67]. Burn injury causes a transient induction of CD14 in Kupffer cells, leading to enhanced TLR4 signaling and cytokine production in mice [68]. This is supported by work with CD14 knockout mice and TLR4 defective animals implicating LPS as the initial inducing agent associated with acute phase responses (APR) in the liver after burn [69]. As a part of the APR, hepatocytes increase production of LBP leading to even greater LPS binding to Kupffer cell TLR4 in rats [70]. Over-stimulated Kupffer cells contribute not only to hepatic damage and steatosis [71], but also to systemic levels of potentially damaging pro-inflammatory cytokines. It is of great clinical importance, therefore, that Kupffer cell sensitivity to post-burn LPS is significantly increased when alcohol intoxication precedes burn injury as seen in a mouse model (Chen, in preparation).

Alcohol has a well described and dynamic influence on the sensitivity of macrophages to LPS, with paradoxical effects depending on the duration of exposure [72]. After a single dose of alcohol (4g/kg), rat Kupffer cells display a brief initial decrease in LPS sensitivity that is reciprocally increased by 24 hours, correlating to a 5-
fold increase in CD14 expression [73]. This is supported by studies using a mouse model of acute intoxication where the activity of interleukin-1 receptor-associated kinase (IRAK), a protein downstream of TLR4, was increased 21 hours after a single dose of alcohol [74]. In contrast to chronic alcoholics, binge drinkers experience only a minor and transient spike of LPS in portal blood [36] and therefore increased Kupffer cell CD14 has limited clinical significance. In the context of a burn however, alcohol-exposed Kupffer cells have abundant and prolonged LPS exposure leading to aberrant increases in both the co-receptor and ligand for TLR4 signaling.

Recent work from our laboratory demonstrated that a single dose of alcohol (1.1 g/kg) potentiated post-burn hepatic damage via Kupffer cell derangement in mice (Chen et al, in preparation). Specifically, Kupffer cells isolated from mice exposed to both alcohol and burn were sensitized to LPS and produced significantly greater amounts of IL-6 in a p38-dependent manner. A member of the mitogen activated protein kinase (MAPK) family, p38 has a well-established role in post burn remote organ damage [75,76,77,78,79], is altered by intoxication [80,81] and plays a role in enhanced TLR4 reactivity following injury [82]. Antecedent depletion of Kupffer cells or global p38 inhibition after intoxication and burn injury in mice attenuated hepatic and pulmonary damage to a similar extent suggesting a prominent role for Kupffer cell p38 (Chen et al, in preparation). Furthermore, both the absence of Kupffer cells and p38 inhibition decreased hepatic damage to a level seen after a burn alone, again pointing to alcohol’s ability to alter the gut-liver axis as a key mechanism for worsening post burn outcome.
Cytokine Production

A paramount consequence of hyperactive Kupffer cells is the excessive release of cytokines. While beneficial at appropriate levels, aberrant cytokine release is a hallmark of the Systemic Inflammatory Response Syndrome (SIRS) and a common complication after burn [83]. The multifunctional cytokine IL-6 is particularly elevated in thermally injured patients [84,85] and correlates to mortality risk after injury [23,84,86,87]. In animal models of intoxication and burn, increased systemic levels of IL-6 are found relative to either alcohol or burn alone [19,21,23] with the highest concentrations found in the liver [21]. Furthermore, systemic IL-6 decreases in the absence of Kupffer cells after intoxication and burn (Chen et al, in preparation) and other models of trauma support the idea that Kupffer cells may be the systemic source of IL-6 after injury [88]. Identifying the systemic source of IL-6 may be crucial to successful treatment of intoxicated burn patients, as recent evidence in animal models revealed excessive IL-6 is responsible for amplified pulmonary inflammation [89] and gut permeability [29,48] in this combined injury setting. These findings do not rule out the influence of other cytokines and signaling pathways which are altered by intoxication and burn, some of which are depicted in Figure 2. However, they implicate alterations in the gut-liver axis, specifically in Kupffer cell IL-6 production, as key mechanisms for the ability of alcohol to worsen post burn hepatic damage and overall outcome.
Figure 2: Potential signaling pathway alterations in Kupffer cells after burn injury and/or alcohol intoxication. activator protein-1 (AP-1), alpha-2a adrenoceptor (α2A-AR), cluster of differentiation 14 (CD14), inhibitor of kappa B (IκB), interleukin-1 receptor-associated kinase (IRAK), interleukin-6 (IL-6), endoplasmic reticulum (ER), lipopolysaccharide (LPS), LPS binding protein (LBP), nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), nuclear factor kappa B (NF-κB), Toll-like Receptor 4 (TLR4), tumor necrosis factor (TNF). Numbers in figure 2 denote associated references. *Chen et al, in preparation.

Oxidative Stress

Another factor contributing to hepatic cytokine production after alcohol or burn is oxidative stress. Hepatic aldehyde dehydrogenase (ALDH) activity is reduced after a burn injury in rats allowing for the accumulation of toxic aldehydes produced by lipid peroxidation [90]. The generated reactive oxygen species (ROS) can directly damage intracellular proteins and lead to activation of multiple inflammatory signaling pathways including MAPKs and NF-κB [91,92]. In addition, antioxidant enzymes in rat livers are
down regulated within 24 hours after a burn [93], increasing the susceptibility of the liver to oxidative stress. Alcohol directly stimulates the release of ROS in Kupffer cells within 3 hours of intoxication as seen in a study of a rat model [94]. Other animal studies implicate this ethanol-induced oxidative stress as a key regulator of Kupffer cell LPS sensitivity after a single dose of alcohol (5g/kg) [95,96]. Furthermore, a byproduct of alcohol metabolism, acetaldehyde, is an example of the aforementioned toxic aldehydes normally cleared by ALDH [90]. Not surprisingly, animal studies revealed alcohol increased post-burn hepatic oxidative stress and IL-6 production in a dose dependent manner [23].

**Steatosis**

The accumulation of triglycerides within hepatocytes, known as steatosis, is a common finding after burn injury with the clinical significance depending on the etiology and severity [97,98]. Animal and clinical studies demonstrate that post burn β-adrenergic peripheral lipolysis increases the delivery of free fatty acids to the liver [99,100,101], which has concomitantly decreased ability for very low-density lipoprotein (VLDL)-triglyceride export [101]. The resulting hepatic fatty infiltration is associated with increased incidence of sepsis and mortality in pediatric burn patients [54], highlighting the importance of organ integrity. Steatosis is also commonly caused by alcohol consumption [102] and the degree of steatosis is substantially increased in mice with the combination of alcohol and burn over either insult alone [20,22]. Animal studies show that alcohol metabolism increases the ratio of NADH:NAD+ which then inhibits β-oxidation of fatty acids while increasing the rate of their esterification [103], leading to
excessive triglyceride storage. Uncontrolled hyperglycemia [104], oxidative stress [105], LPS signaling [106] and Kupffer cell cytokine secretion [71] can also contribute to steatosis development. As these factors are all influenced by both alcohol and burn (Figure 3), they may represent potential mechanisms by which intoxication potentiates post burn steatosis.

Figure 3: Mechanisms of steatosis development after burn injury and/or alcohol intoxication. Acetyl coenzyme A (acetyl CoA), glycerol 3-phosphate (glycerol 3-p), nicotinamide adenine dinucleotide (NAD), triglyceride (TG), very low-density lipoprotein (VLDL)
Conclusions

Intoxication plays a detrimental role in traumatic injury as both a causative agent and complicating factor in recovery. This is dramatically observed in burns where high prevalence and established consequences warrant further investigation into the mechanisms by which alcohol worsens the post burn response. Furthermore, the very presence of alcohol, regardless of dependence or pre-existing hepatic sequelae, potentiates post burn hepatic damage. Clinical and animal studies suggest alcohol alters the post burn gut-liver axis in the early phases after injury which may lower the threshold for longer term hepatic derangement and hypermetabolism. As our understanding of this combined injury grows, more targeted therapeutic approaches may be possible.
CHAPTER 3
AN ALTERATION IN THE GUT-LIVER AXIS DRIVES PULMONARY INFLAMMATION AFTER INTOXICATION AND BURN

Abstract

Approximately half of all adult burn patients are intoxicated at the time of their injury and have worsened clinical outcomes compared to those without prior alcohol exposure. This study tested the hypothesis that intoxication alters the gut-liver axis leading to increased pulmonary inflammation mediated by burn-induced IL-6 in the liver. To this end, C57BL/6 mice were given 1.2 g/kg ethanol 30 minutes prior to a 15% total body surface area burn. To restore gut barrier function, a specific myosin light chain kinase inhibitor [membrane-permeant inhibitor of kinase (PIK)] was administered 30 minutes after injury which we have demonstrated to reduce bacterial translocation from the gut. Limiting bacterial translocation with PIK attenuated hepatic damage as measured by a 47% reduction in serum alanine aminotransferase (p<0.05) as well as a 33% reduction in hepatic IL-6 mRNA expression (p<0.05) compared to intoxicated burned mice without PIK. This mitigation of hepatic damage was associated with a 49% decline in pulmonary neutrophil infiltration (p<0.05) and decreased alveolar wall thickening compared to matched controls. These results were reproduced by prophylactically
reducing the bacterial load in the intestines with oral antibiotics before intoxication and burn. Overall these data suggest the gut-liver axis is deranged when intoxication precedes burn and that limiting bacterial translocation in this setting attenuates hepatic damage and pulmonary inflammation.

**Introduction**

As the most frequently abused substance in the United States, alcohol has a high association with unintentional injuries [2,3] and is the third leading cause of preventable death [1]. The practice of consuming enough alcohol to bring the blood alcohol concentration (BAC) to 0.08% or above, known as binge drinking, is an increasingly prevalent form of intoxication [4] and the characteristic consumption pattern among patients presenting with traumatic injury, including burns [5]. Approximately half of adult burn patients are intoxicated at the time of admission and these patients have worse clinical outcomes than individuals who sustain similar injuries not under the influence [6,7,8]. Specifically, they are twice as likely to acquire an infection, undergo 60% more surgical procedures, require more days on mechanical ventilation, and stay longer in the intensive care unit [6,9]. As a result of these complications, burn patients with prior alcohol exposure have been identified as a patient population at high risk for death in the early stages post burn [10]. With 1 million burn injuries in the US each year, resulting in 40,000 hospitalizations [11], this represents a substantial burden of morbidity, mortality and socioeconomic cost that is worsened by alcohol abuse. Despite the prevalence and known repercussions of being intoxicated at the time of a burn injury, there is little difference in the treatment and management of burn patients with and without prior
alcohol exposure. In fact, relatively little is known about the mechanisms by which alcohol affects the post burn response. The complicated natural history of burn injuries as well as the complex and duration-dependent effects of alcohol impact nearly every major organ system [21]. Therefore, the detrimental response to intoxication and burn likely encompasses aberrations, and potentially crosstalk, between multiple organs.

The studies reported herein examine the role of the gut-liver axis after intoxication and burn by limiting bacterial translocation through pharmacologic restoration of intestinal barrier function after injury. This was accomplished by administration of an inhibitor of myosin light chain kinase (MLCK) after a burn, which we have previously demonstrated decreases intestinal barrier damage and subsequent bacterial translocation in this setting [29]. In a separate group of animals, the initial bacterial burden within the intestines was decreased before injury by prophylactic oral antibiotics. Finally, systemic effects of manipulating the gut-liver axis were investigated by examining pulmonary inflammation and interleukin-6 levels.

**Materials & Methods**

**Animals**

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in sterile micro-isolator cages under specific pathogen-free conditions in the Loyola University Chicago Comparative Medicine Facility. All experiments were conducted with approval of and strict accordance to the Loyola Institutional Animal Care and Use Committee. All experiments were performed with mice weighing between 23-25
and during the hours of 8-10 AM to avoid confounding factors related to circadian rhythms.

**Murine Model of Intoxication and Burn Injury**

A murine model of a single binge ethanol exposure and burn injury was employed as described previously [107] with minor modifications. Briefly, mice were given a single intraperitoneal (i.p.) dose of ethanol (1.12 g/kg) that resulted in a blood ethanol concentration of 150–180 mg/dl at 30 minutes or saline vehicle. The mice were then anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), their dorsum shaved, and placed in a plastic template exposing 15% of the total body surface area and subjected to a scald injury in a 90-92°C water bath or a sham injury in room-temperature water. The scald injury resulted in an insensate, full-thickness burn [108]. The mice were then resuscitated with 1.0 ml saline and allowed to recover on warming pads. The intoxicated and burn injured mice were split into two groups and at 30 minutes after injury, given the specific long MLCK inhibitor PIK (membrane-permeant inhibitor of kinase,100μl i.p. of 50 μM) or saline control as described previously [29]. A separate group of mice received prophylactic antibiotics (neomycin 100 mg/kg/day and polymyxin E (60mg/kg/day) via drinking water for 10 days before intoxication and burn. This resulted in a 3.67 log reduction in culturable aerobic fecal bacteria at the time of injury. Mice were sacrificed by CO₂ narcosis followed by exsanguination 24 hours after injury. The control arms of sham vehicle, sham ethanol, and burn vehicle are presented herein to demonstrate baseline results and provide injury context for the assessed parameters. The data from these control groups can be found in the manuscript figures and are consistent with
previously reported results in the intestines [29], liver [23], and lungs[89]. As this study examines a therapeutic intervention for the established consequences of intoxication and burn injury in this animal model, the discussion of this study will focus on the animals given the combined injury of ethanol and burn, with and without treatments.

**Histopathologic Examination of the Ileum.**

At 6 and 24 hours post-injury, mice were euthanized by CO\textsubscript{2} narcosis and the ileum was harvested and fixed overnight in 10% formalin. Samples were then embedded in paraffin, sectioned at 5 microns, and stained with hematoxylin and eosin (H&E). Images were taken at 200x.

**Immunofluorescent Staining in the Ileum**

Immunofluorescent staining was done as previously described [29] with minor modifications. Briefly, a small section of ileum (5 mm) was embedded in optimal cutting temperature (OCT) media and frozen at -80ºC. The ileum was sectioned (8 μm) and stained with rabbit anti-ZO-1 (Invitrogen, Carlsbad, CA) followed by goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Sections were further stained with fluorescent-conjugated phalloidin (actin) and Hoechst nuclear stain (Invitrogen Carlsbad, CA). Images were taken at 400x magnification.

**Bacterial Translocation**

Bacterial translocation was assessed as previously described [29,48]. Briefly, 3–5 mesenteric lymph nodes (MLN) per mouse were removed and placed in cold RPMI, and kept on ice. MLN were separated from connective tissue and homogenized in RPMI
using frosted glass slides. Homogenates were plated in triplicate on tryptic soy agar and placed in a 37°C incubator overnight.

Histopathologic Examination of the Liver

The whole liver was removed at the time of sacrifice, weighed and embedded in OCT media or snap frozen in liquid nitrogen. Frozen sections were cut at 7 μm, stained with Oil Red O, and then examined for the presence of fat droplets as described previously [22]. Briefly, sectioned tissue was rinsed in isopropyl alcohol and then placed in a filtered working solution of Oil Red O for 20 minutes. The slides were then rinsed in isopropyl alcohol, followed by tap water and counterstained with hematoxylin for 1 minute. The slides were then serially rinsed in tap water, ammonia water (lithium carbonate), and finally tap water. Representative images were taken at 400x magnification.

Liver Cytokines and Triglyceride Quantification

Liver tissue was homogenized in 1 ml of BioPlex cell lysis buffer (BioRad, Hercules, CA) and analyzed for cytokine production using an enzyme-linked immunosorbent assay (ELISA) for interleukin-6 (IL-6; BD Biosciences, Franklin Lakes, NJ). The results were normalized to total protein using the BioRad protein assay (BioRad, Hercules, CA). A portion of the frozen liver was used in a Triglyceride Quantification Kit according to manufacturer instruction (Abcam, Cambridge, MA).

Hepatic IL-6 mRNA Expression

RNA was isolated from liver homogenate using RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA was produced using iScript cDNA synthesis kit (Bio-Rad,
Hercules, CA). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the StepOne Plus Real-Time PCR Systems (Applied Bio-Systems, Carlsbad, CA) and data analyzed by the 2-ddCT relative quantification method [109].

Serum Measurements

At 24 hours post-injury mice were euthanized, blood was collected via cardiac puncture and harvested for serum by centrifugation after clotting. Serum aliquots were used to measure IL-6 by ELISA (BD Biosciences, Franklin Lakes, NJ) or liver transaminase levels using a DRI-CHEM 7000 (HESKA, Loveland, CO) as described previously [19].

Histopathologic Examination of the Lungs

The upper right lobe of the lung was inflated with 10% formalin and fixed overnight as described previously [110]. The lung was then embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). The sections were analyzed microscopically in a blinded fashion for the number of neutrophils in 10 high power fields as a marker of inflammation. Representative images were taken at 400x magnification. Ten high power fields (400X) per animal were analyzed using the Java-based imaging program ImageJ (National Institutes of Health, Bethesda, MD). The images were converted to binary to differentiate lung tissue from air space and then analyzed for the percent area covered by lung tissue in each field of view as described previously [89].
KC Analysis of Lung Homogenates

The right middle lung lobe was snap-frozen in liquid nitrogen. The tissues were then homogenized in 1 ml of BioPlex cell lysis buffer according to manufacturer’s instructions (BioRad, Hercules, CA). The homogenates were filtered and analyzed using an ELISA for KC (BD Biosciences, Franklin Lakes, NJ), a neutrophil chemokine and murine analog for human IL-8. The results were normalized to total protein using the BioRad protein assay (BioRad, Hercules, CA).

Statistical Analysis

Statistical comparisons (GraphPad Instat) were made between intoxicated burned mice given saline and intoxicated burned mice given a treatment (PIK or antibiotics) using a Student's t-test and Tukey's post hoc test (p<0.05). Data are reported as mean values ± the standard error of the mean (SEM).

Results

Limiting Bacterial Translocation Attenuates Hepatosteatosis after Intoxication and Burn

Twenty-four hours after intoxication and burn, intestinal villi demonstrated a loss of tight junction integrity (Figure 4A), a 62-fold increase in bacterial translocation (Figure 4B), and a 14-fold elevation in hepatic triglycerides (Figure 4C) compared to sham injured animals. PIK treatment after intoxication and burn restored tight junction complexes as demonstrated by colocalization of ZO-1 and actin (Figure 4A). This corresponded to a 71% decrease (p<0.05) in bacterial translocation (Figure 4B), and a 75% reduction (p<0.05) in liver triglycerides (Figure 4C) compared to saline-treated
Figure 4. Decreasing bacterial translocation attenuated hepatosteatosis. (A) Tight junction protein localization (400x). zonula occludens protein-1 (ZO-1; green), phalloidin (red) and nuclei (blue). (B) Bacterial translocation. colony forming units (CFU). (C) Liver sections with ORO (400x). (D) Hepatic triglycerides. *p<0.05 compared to PIK and Abx by Student’s T test. N=4-6 animals per group. _____ represents sham vehicle, ______ represents sham ethanol, and _____ represents burn vehicle.
animals undergoing the same injury. Antibiotic prophylaxis failed to restore tight junction complexes in the terminal ileum (Figure 4A) but did reduce bacterial translocation by 93% (p<0.05) (Figure 4B) which corresponded to an 84% decrease (p<0.05) in liver triglycerides (Figure 4C) relative to intoxicated and burn injured mice given saline. The attenuation of steatosis after intoxication and burn in PIK or antibiotic-treated animals was evident upon histologic examination in Oil Red O-stained images (Figure 4D).

PIK or Antibiotic Treatment Reduces Hepatic Damage After Intoxication and Burn

In comparison to sham injured animals, mice given ethanol and burn were found to have a >25-fold increase in serum AST (Figure 5A) and ALT (Figure 5B) as well as 1.3-fold increase in liver weight to body weight ratio (Figure 5C). PIK treatment after the combined insult alleviated hepatic damage as measured by a 49% reduction (p<0.05) in serum AST, a 47% decrease (p<0.05) in serum ALT and a 24% lower (p<0.05) liver weight to body weight ratio compared to intoxicated and burned animals given saline (Figure 2 A-C). Analogous to PIK treatment, antibiotic prophylaxis reduced serum AST by 44% (p<0.05), ALT by 43% (p<0.05) and liver weight to body weight ratio by 23% (p<0.05), relative to animals given saline after ethanol and burn (Figure 5A-C).

Hepatic and Systemic Levels of IL-6 are Reduced by Limiting Bacterial Translocation after Intoxication and Burn

Twenty-four hours after intoxication and burn, an approximate 60-fold increase in hepatic mRNA expression (Figure 6B) corresponded to a 10-fold increase in IL-6 protein
Figure 5. Liver damage 24 hours after intoxication and burn is diminished when bacterial translocation is inhibited. (A) Serum levels of aspartate aminotransferase (AST) after treatment with saline vehicle, MLCK inhibition (PIK), or prophylactic antibiotics (Abx). (B) Serum levels of alanine aminotransferase (ALT). (C) Liver weight to total body weight ratio. *p<0.05 compared to PIK and Abx treatments by Student’s T test. N=4-6 animals per group. ______ represents sham vehicle, ______ represents sham ethanol, and ______ represents burn vehicle.
levels in the liver (Figure 6A) and serum (Figure 6C) compared to sham-injured mice. After the combined insult of intoxication and burn, PIK treatment led to a 33% reduction (p<0.05) in hepatic mRNA expression (Figure 6B), a 71% decrease (p<0.05) in IL-6 protein levels in the liver (Figure 6A) and 70% lower (p<0.05) serum IL-6 (Figure 6C). Similarly, IL-6 levels in mice that received antibiotic prophylaxis reduced hepatic IL-6 by 51% (p<0.05), IL-6 mRNA expression by 45% (p<0.05), and serum IL-6 by 68% (p<0.05) compared to mice undergoing intoxication and burn given saline.

Figure 6. Interleukin-6 production is decreased with PIK or antibiotic treatment. (A) Hepatic IL-6. (B) IL-6 mRNA expression levels in liver. (C) Serum IL-6 levels. *p<0.05 vs PIK and Abx by Student’s T test. N=4-6 animals per group. ______ represents sham vehicle, _______ represents sham ethanol, and _____ represents burn vehicle.
Pulmonary Inflammation after Intoxication and Burn is Attenuated with PIK or Antibiotics

Intoxication and burn substantially increased alveolar wall thickness by visual examination (Figure 7A) compared to sham injured animals. Imaging software was employed to quantify the amount of lung tissue versus air space as a measure of alveolar wall thickness as described previously [19]. A 3-fold increase in lung tissue area was observed in intoxicated and burned mice compared to those receiving a sham injury (Figure 7B) which was accompanied by a >10-fold increase in neutrophil infiltration (Figure 7C) and pulmonary KC levels (Figure 7D). PIK treatment as well as antibiotics lessened pulmonary congestion and cellularity upon visual examination (Figure 7A). Consistent with visual findings, PIK treatment decreased pulmonary congestion by 23% (p<0.05) compared to matched controls and similarly, antibiotic prophylaxis lessened congestion by 29% (p<0.05) (Figure 7B). Neutrophil accumulation was reduced by 49% (p<0.05) with PIK treatment and 43% (p<0.05) with antibiotics compared to intoxicated burned mice given saline (Figure 7C). Finally, levels of the neutrophil chemokine, KC, were measured in lung homogenates and were reduced by 48% (p<0.05) and 41% in PIK and antibiotic treated animals respectively compared to intoxicated and burn injured mice given saline after injury (Figure 7D).
Figure 7. Pulmonary inflammation 24 hours after intoxication and burn is attenuated by restoration of the gut-liver axis. (A) Lung histology (hematoxylin and eosin at 400x) of intoxicated and burn-injured mice receiving PIK treatment or prophylactic antibiotics (Abx). (B) Pulmonary congestion quantified using imaging software to calculate the lung tissue area in 10 fields of view. (C) Neutrophil (PMN) quantification in 10 high power fields of view of intoxicated and burn-injured mice. (D) Pulmonary KC levels. *p<0.05 compared to PIK and antibiotic (Abx) treatments by Student’s T test. N=4-6 animals per group. ______ represents sham vehicle, ______ represents sham ethanol, and ______ represents burn vehicle.
Summary

The studies above demonstrate a clear and reversible interaction between bacterial translocation from the intestines and the hepatic response to burn injury and ethanol exposure. Interestingly, both PIK and antibiotic treatments reduced the amount of bacterial translocation to the extent observed after a burn alone (Figure 4B, [19,29,48]) and the subsequent responses seen in the liver and lungs matched the degree observed in post burn injuries without ethanol exposure (Figures 5 & 7, [19,89,111]). These results may suggest that intoxication worsens post burn outcome through alterations in the gut-liver axis since the additional damage observed with antecedent ethanol exposure can be reversed by restoring gut-liver crosstalk to levels seen after a burn alone. Clinically, there are no accurate predictors for increased burn risk amongst alcohol abusers and indiscriminate antibiotic prophylaxis in this population may raise numerous issues with antimicrobial stewardship, drug compliance, altering endogenous flora, drug interactions and cost. Therefore, an ideal therapeutic in this setting could be administered after the injury. PIK is still an experimental research drug and unfortunately there are currently no licensed drugs that act on MLCK inhibition to our knowledge. However we believe our data demonstrates a proof of concept that can hopefully be taken into human trials as drugs become available.
CHAPTER 4

KUPFFER CELL P38 MAPK SIGNALING DRIVES POST BURN HEPATIC DAMAGE AND PULMONARY INFLAMMATION WHEN ALCOHOL INTOXICATION PRECEDES BURN INJURY

Abstract

The widespread and increasing trend of binge drinking is accompanied by a concomitant rise in the prevalence of trauma patients intoxicated at the time of injury. Nearly half of adult burn patients are intoxicated at the time of admission and the presence of alcohol is an independent risk factor for death in the early stages post burn. We recently reported hepatic IL-6 drives pulmonary inflammation in this setting and we now seek to determine the role of Kupffer cells in this aberrant response. To this end, mice were given ethanol (1.2g/kg) by oral gavage 30 minutes prior to a 15% burn injury. Kupffer cells were isolated 24 hours after injury and analyzed for p38 activity and IL-6 production. In separate experiments, Kupffer cell depletion with clodronate liposomes or p38 inhibition was achieved via intraperitoneal injection of SB203580. Kupffer cells of intoxicated burned mice had a 2-fold (p<0.05) elevation of p38 activation relative to burn alone and this corresponded to a 43% (p<0.05) increase in IL-6 production. Kupffer cell depletion attenuated hepatic damage as seen by decreases of 53% (p<0.05) in serum ALT and 74% (p<0.05) in hepatic triglycerides, as well as a 77% reduction (p<0.05) in serum IL-6 levels compared to matched controls. This mitigation of hepatic damage was
associated with a 54% decrease (p<0.05) in pulmonary neutrophil infiltration and reduced alveolar wall thickening by 45% p<0.05), suggesting a crucial role for Kupffer cells in both hepatic and pulmonary damage in this setting. In vivo p38 inhibition conferred nearly identical hepatic and pulmonary protection after the combined injury as mice depleted of Kupffer cells, highlighting the role of Kupffer cell p38 in causing injury. Taken together, these data suggest intoxication exacerbates post burn hepatic damage through p38-dependent IL-6 production in Kupffer cells which may serve as a potential therapeutic target in this common clinical scenario.

Introduction

Severe burns are a devastating injury affecting every major organ system, with the degree of systemic inflammation correlating to the size of the burn [17]. Advances in wound care, fluid resuscitation and infection control have dramatically increased survival rates [112], though hepatic derangements and a hypermetabolic state can persist for years after the initial injury [24]. The physiologic response to a burn depends on a variety of factors including the age of the patient, the size and severity of the burn, and the presence of alcohol [10]. Nearly 50% of patients admitted for burns have a positive blood alcohol content at presentation and these patients have worse clinical outcomes than individuals who sustain similar injuries not under the influence of alcohol [6,7,8]. Specifically, they are twice as likely to acquire an infection, require more surgical procedures and have a longer duration of stay in the intensive care unit than their nonintoxicated counterparts [6,9]. As a result of these complications, burn patients with prior alcohol exposure have been identified as a patient population at high risk for death
in the early stages post burn [10]. However, few differences currently exist in their treatment and management despite the established consequences. With 1 million burn injuries in the US each year, resulting in 40,000 hospitalizations [11], this represents a substantial burden of morbidity, mortality and socioeconomic cost that is worsened by alcohol abuse. Binge drinking in particular is an increasingly prevalent form of intoxication [4] and is the characteristic pattern of drinking among patients presenting with traumatic injury, including burns [5]. Recent studies suggest the majority of intoxicated burn patients are binge drinkers without evidence of chronic alcoholism [5], consistent with the majority of alcohol consumption in the US [4]. The increasing prevalence of binge drinking and its high association with traumatic injury in an otherwise healthy population warrant further investigation into the mechanism by which alcohol worsens the post burn response.

As a vital organ with metabolic, hormonal and immune functions, the liver is central in the response to burn injury and plays a pivotal role in post-burn outcome [14]. This is highlighted by the close correlation between hepatic function and prognosis after burn [15]. The liver is also the major site of alcohol metabolism and toxicity and we have previously demonstrated that intoxication potentiates post burn hepatic damage [19,20,22,23]. Furthermore, we reported that increased hepatic interleukin-6 (IL-6) drives pulmonary inflammation in the setting of this combined injury [20,89], making it essential to understand the source and underlying mechanisms of its production. Animal studies demonstrate that post burn intestinal permeability is exacerbated when intoxication precedes the injury [32,33], leading to greater translocation of bacteria and
bacterial products such as lipopolysaccharide (LPS) into the lymphatic [45,46,48] and portal [47] systems. Within the liver, Kupffer cells line the hepatic sinusoids and sample the portal blood for foreign antigen. Upon activation by LPS, Kupffer cells release pro-inflammatory cytokines which orchestrate an appropriate hepatic response [60]. In an injury context, however, Kupffer cells can become hyperactive and release excessive levels of cytokines, including IL-6, causing hepatic and systemic damage [64,67]. Independently, both alcohol [73,96] and burn [67,113] are known to increase Kupffer cell sensitivity to LPS through Toll-like receptor 4 (TLR4) signaling and downstream p38 mitogen activated protein kinase (MAPK). We therefore sought to examine the role of Kupffer cells in the response to intoxication and burn. We describe within findings that demonstrate intoxication augments Kupffer cell IL-6 production through increased p38 activation after burn.

**Materials & Methods**

**Animals**

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in sterile micro-isolator cages under specific pathogen-free conditions in the Loyola University Chicago Comparative Medicine Facility. All experiments were conducted with approval of and strict accordance to the Loyola Institutional Animal Care and Use Committee. All experiments were performed with mice weighing between 23-25 g and during the hours of 8-10 AM to avoid confounding factors related to circadian rhythms.
Murine Model of Intoxication and Burn Injury

A murine model of a single binge ethanol exposure and burn injury was employed as described previously [107] with minor modifications. Briefly, mice were given a single dose of ethanol (1.12 g/kg) by oral gavage that resulted in a blood alcohol concentration of approximately 180 mg/dl at 30 minutes [19]. The mice were then anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), their dorsum shaved and they were placed in a plastic template exposing 15% of the total body surface area (TBSA) and subjected to a scald injury in a 92°C water bath or a sham injury in room-temperature water. The scald injury resulted in an insensate, full-thickness burn [108]. The mice were then resuscitated with 1.0 ml of 0.9% normal saline and placed on warming pads until recovered from anesthesia. Mice were euthanized by carbon dioxide narcosis followed by exsanguination 24 hours after injury. For experiments involving in vivo p38 inhibition, the intoxicated and burn injured mice were split into two groups and at 30 minutes after injury, given an intraperitoneal injection of the selective p38 MAPK inhibitor, SB203580 (InvivoGen, 10mg/kg) or vehicle control (saline). SB203580 at this dose has been previously demonstrated to specifically inhibit Kupffer cell p38 MAP kinase 24 hours after burn injury ([113], Fig. S1). For experiments involving antecedent Kupffer cell depletion, clodronate liposomes (Encapsula NanoSciences, 0.5mg/kg) or vehicle control (empty liposomes) were administered via tail vein injection. This method of administration has been shown to cause Kupffer cell depletion while limiting the effects on other macrophage populations ([114], Fig. S1). The control arms of sham vehicle, sham ethanol, and burn vehicle are presented herein for the clodronate and p38i
experiments to demonstrate baseline results and provide injury context for the assessed parameters. The data from these control groups can be found in the manuscript figures and are consistent with previously reported results in the liver [19,20,23], and lungs [19,34,89]. As this study examines the role of Kupffer cells in the established consequences of intoxication and burn injury in this animal model, the discussion of the in vivo studies will focus on the animals given the combined injury of ethanol and burn, with and without treatments. Furthermore, as the treatment vehicle controls given to intoxicated and burn injured mice (saline and empty liposomes for p38 inhibition and clodronate, respectively) did not differ from intoxication and burn without vehicle controls, only one set of results are shown for clarity.

Blood and Serum Measurements

At 24 hours post injury, mice were euthanized, blood was collected via cardiac puncture, an aliquot was placed into a microcapillary tube, and read for a complete blood count with differential by Hemavet (Drew Scientific, Dallas, TX). The remaining blood was harvested for serum by centrifugation after clotting. Serum aliquots were used to measure IL-6 by ELISA (BD Biosciences, Franklin Lakes, NJ) or liver transaminase levels using a DRI-CHEM 7000 (HESKA, Loveland, CO) as described previously [19].

Histologic Examination of the Liver

The whole liver was removed at the time of sacrifice, weighed and a portion was embedded in OCT media or snap frozen in liquid nitrogen. Frozen sections were cut at 7 μm, stained with Oil Red O, and then examined for the presence of fat droplets as described previously [22]. Briefly, sectioned tissue was rinsed in isopropyl alcohol and
then placed in a filtered working solution of Oil Red O for 20 minutes. The slides were then rinsed in isopropyl alcohol, followed by tap water and counterstained with hematoxylin for 1 minute. The slides were then serially rinsed in tap water, ammonia water (lithium carbonate), and finally tap water. Representative images were taken at 400x magnification.

Assessment of Liver Cytokines and Triglyceride Quantification

Liver tissue was homogenized in 1 ml of BioPlex cell lysis buffer according to manufacturer’s instructions (BioRad, Hercules, CA) and analyzed for cytokine production using an ELISA for interleukin-6 (IL-6; BD Biosciences, Franklin Lakes, NJ). The results were normalized to total protein using the BioRad protein assay (BioRad, Hercules, CA). A portion of the frozen liver was used in a Triglyceride Quantification Kit according to manufacturer instruction (Abcam, Cambridge, MA).

Isolation of Kupffer Cells

Kupffer cells were isolated 24 hour after injury as described by Kuriakose et al. [115] with minor modifications. Briefly, rinsed liver tissue was incubated at 37°C for 30 minutes in collagenase IV (5000 CDU/mL) and run through an automated gentleMACS Dissociator (Miltenyi Biotec, Auburn, CA) before being passed through a sterile 100um strainer into phosphate-buffered saline (Miltenyi Biotec, Auburn, CA) containing 0.5% bovine serum albumin. The hepatocytes were then removed by low-speed centrifugation at 20xg for 4 minutes, and the residual cell suspension was centrifuged twice at 300xg for 10 minutes at 4°C with the cell pellet washed with Red Blood Cell Lysis Solution between centrifugations. CD11b+ cells were then enriched by positive selection using
AUTOMACS (Miltenyi Biotec Auburn, CA). Enriched liver CD11b+ cells isolate by this method have been shown to be greater than 96% positive for F4/80 expression as assessed by flow cytometry.

Cell Culture

Isolated Kupffer cells were cultured at a concentration of $3 \times 10^5$ per well in RPMI medium supplemented with 10% fetal bovine serum and 50µg/ml gentamycin for 2 hours. Cells were pretreated with SB203580 (10uM) for 1 hour before stimulation with LPS (100ng/mL) followed by incubation at 37°C with 5% CO$_2$ and 95% humidity for 18 hours. At the concentration used, SB203580 is highly selective for p38 [116]. Supernatants were removed and analyzed for cytokine production using an enzyme-linked immunosorbent assay (ELISA) for interleukin-6 (IL-6; BD Biosciences, Franklin Lakes, NJ). Activation of p38 was determined by cell-based ELISA (RayBiotech Inc, Norcross, GA) using primary antibodies against p38 and phospho-p38 (Thr180/Tyr182) as specified per manufacturer instructions.

Histologic Examination of the Lungs

The upper right lobe of the lung was inflated with 10% formalin and fixed overnight as described previously [110]. The lung was then embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). The sections were analyzed microscopically in a blinded fashion for the number of neutrophils in 10 high power fields as a marker of inflammation. Representative images were taken at 400x magnification. Ten high power fields (400X) per animal were analyzed using the Java-based imaging program ImageJ (National Institutes of Health, Bethesda, MD). The
images were converted to binary to differentiate lung tissue from air space and then analyzed for the percent area covered by lung tissue as described previously [89].

Statistical analysis

Statistical comparisons (GraphPad Instat) were made between the sham vehicle, sham ethanol, burn vehicle, and burn ethanol animals, resulting in four total groups analyzed. One-way analysis of variance (ANOVA) was used to determine differences between groups, and Tukey's post hoc test once significance was achieved (p<0.05). Statistical comparisons made between intoxicated burned mice given saline and intoxicated burned mice given a treatment (clodronate or SB203580) were performed using a Student's t-test (p<0.05). Data are reported as mean values ± the standard error of the mean (SEM).

Results

Kupffer Cell p38 Activation and IL-6 Production

Total p38 was detectable in all samples and did not change between treatment groups. Ethanol intoxication or burn injury alone increased Kupffer cell p38 activation approximately 2-fold (p<0.05) over nonintoxicated sham injured animals (Figure 8A), corresponding to an approximate 2-fold increase in IL-6 production (Figure 8B) after LPS stimulation. The combination of ethanol and burn injury led to a 4-fold (p<0.05) increase in p38 activation relative to sham, which was 2-fold (p<0.05) greater than either insult alone. IL-6 levels paralleled p38 activation with the combined injury demonstrating a 3-fold (p<0.05) and 1.5-fold (p<0.05) increase over sham injury or isolated intoxication or burn, respectively (Figure 8B). Ex vivo p38 inhibition abrogated
the increases in LPS-induced p38 activation (Figure 8A) or IL-6 production (Figure 8B) in Kupffer cells from any injury group.

Figure 8. Kupffer cell p38 activation is increased when intoxication precedes burn. Kupffer cells were isolated 24 hours after injury and assessed for (A) p38 activation and (B) IL-6 production after LPS-stimulation with and without the presence of the p38 inhibitor SB203580 (p38i). *p<0.05 compared to all groups given LPS without inhibitor. #p<0.05 compared to Sham Vehicle given LPS without inhibitor. N=4-6 animals per group.

Kupffer Cell Depletion or p38 Inhibition Attenuates Hepatic Damage after Intoxication and Burn

In comparison to sham injured animals, mice given ethanol and burn were found to have a >15-fold increase in serum AST (Figure 9A) and ALT (Figure 9B) as well as 1.4-fold increase in liver weight to body weight ratio (Figure 9C) which corresponded to 12-fold (p<0.05) greater triglyceride accumulation (Figure 9D). Inhibition of p38 after the combined insult alleviated hepatic damage as measured by a 35% reduction (p<0.05) in serum AST, a 58% decrease (p<0.05) in serum ALT and a 22% lower (p<0.05) liver weight to body weight ratio compared to intoxicated and burned animals given saline.
Figure 9. The absence of Kupffer cells or p38 inhibition equally attenuate hepatic damage. (A) Serum levels of aspartate aminotransferase (AST) after treatment with vehicle, clodronate liposomes (CLO) or SB203580 (p38i). (B) Serum levels of alanine aminotransferase (ALT). (C) Liver weight to total body weight ratio (LW:BW). (D) Hepatic triglycerides. (E) Liver sections stained with Oil Red O at 400x magnification. *p<0.05 compared to CLO and p38i by Student’s T test. N=4-6 animals per group. Represents Sham Vehicle, **Represents Sham Ethanol, and ***Represents Burn Vehicle.
(Figure 9A-C). Analogous to SB203580 treatment, antecedent Kupffer cell depletion reduced serum AST by 44% (p<0.05), serum ALT by 53% (p<0.05) and liver weight to body weight ratio by 28% (p<0.05), relative to animals given saline after ethanol and burn (Figure 9A-C). The decreased hepatic damage after p38 inhibition or the absence of Kupffer cells paralleled decreases in hepatic triglycerides of 59% (p<0.05) and 74% (p<0.05), respectively (Figure 9D). The attenuation of steatosis after intoxication and burn in SB203580 or clodronate-treated animals was evident upon histologic examination in Oil Red O-stained images (Figure 9E).

Hepatic and Systemic Levels of IL-6 are Reduced by Limiting p38 Signaling in Kupffer Cells after Intoxication and Burn

Twenty-four hours after intoxication and burn, a 20-fold increase in IL-6 protein levels in the liver (Figure 10A) and serum (Figure 10C) were observed compared to sham-injured mice. After the combined insult of intoxication and burn, p38 inhibition led to a 57% reduction (p<0.05) in hepatic IL-6 levels (Figure 10A) which corresponded to a 68% decrease (p<0.05) in serum IL-6 (Figure 10C). Similarly, IL-6 levels in mice that lacked Kupffer cells at the time of injury had reduced hepatic IL-6 by 65% (p<0.05) and serum IL-6 by 77% (p<0.05) compared to mice undergoing intoxication and burn with intact Kupffer cells.
Figure 10. Interleukin-6 production is decreased with clodronate liposomes (CLO) or SB203580 (p38i) after intoxication and burn. (A) Interleukin-6 (IL-6) protein levels in liver homogenates. (B) Serum IL-6 levels. *p<0.05 compared to CLO and p38i by Student’s T test. N=4-6 animals per group. ■■■ represents Sham Vehicle, □□□□□ represents Sham Ethanol, and □□□□□□ represents Burn Vehicle.

Pulmonary Inflammation after Intoxication and Burn is Attenuated with SB203580 or Clodronate

Intoxication and burn substantially increased alveolar wall thickness by visual examination (Figure 11A) compared to sham injured animals. Imaging software (ImageJ) was employed to quantify the amount of lung tissue versus air space as a measure of alveolar wall thickness as described previously [19]. A 3-fold increase in lung tissue area was observed in intoxicated and burned mice compared to those receiving a sham injury (Figure 11B) which was accompanied by an 8-fold increase in neutrophil infiltration (Figure 11C). Clodronate or inhibition of p38 lessened pulmonary congestion and cellularity upon visual examination (Figure 11A). Consistent with visual findings, p38 inhibition decreased pulmonary congestion by 43% (p<0.05) compared to matched controls and similarly, Kupffer cell depletion lessened congestion by 45% (p<0.05) (Figure 11B). Neutrophil accumulation was reduced by 58% (p<0.05) with
SB203580 treatment and 54% (p<0.05) with clodronate compared to matched controls (Figure 11C).

Figure 11. Pulmonary inflammation after intoxication and burn is attenuated by Kupffer cell depletion or p38 inhibition. (A) Lung histology (hematoxylin and eosin at 400x) of intoxicated and burn injured mice receiving clodronate liposomes (CLO) or SB203580 (p38i). (B) Neutrophil (PMN) quantification in 10 high power fields of view of intoxicated and burn-injured mice. (C) Pulmonary congestion quantified using imaging software to calculate the lung tissue area in 10 fields of view. *p<0.05 compared to CLO and p38i by Student’s T test. N=4-6 animals per group. ______ represents Sham Vehicle, ______ represents Sham Ethanol, and ______ represents Burn Vehicle.

**In Vivo Treatment Efficacy**

Liver sections of mice administered clodronate liposomes (Figure 12B) exhibited depletion of Kupffer cells as demonstrated by the low CD68 staining compared to the livers of mice administered empty liposomes (Figure 12A). In contrast no differences were found in numbers of circulating monocytes (Figure 12C) or alveolar macrophages (Figure 12D-E). **In vivo** SB203580 treatment abrogated LPS-induced p38 activation in
Kupffer cells isolated from intoxicated and burn injured mice (Figure 12F). Despite an *in vitro* study reporting SB203580 specifically inhibits p38 activity but not p38 activation [117], inhibition of p38 phosphorylation after SB203580 administration is widely reported and used as a mechanism to verify *in vivo* efficacy [75,78,113,118,119]. Inhibition of p38 was confirmed through parallel decreases in LPS-induced IL-6 production (Figure 12G).

Figure 12. *In vivo* Kupffer cell depletion and p38 inhibition. (A) Liver sections stained for CD68 from mice receiving empty liposomes or (B) clodronate liposomes. (C) Peripheral blood monocytes. (D) Lung sections stained for alveolar macrophage marker SiglecF in mice receiving empty liposomes or (E) clodronate liposomes. (F) p38 activation in isolated Kupffer. (G) IL-6 production in isolated Kupffer cells. *p<0.05* compared to all groups by one-way ANOVA. N=3-6 animals per group.
Summary

The studies above delineate a clear and reversible role for Kupffer cell p38 MAPK in the hepatic response to intoxication and burn. Kupffer cells isolated after the combined injury demonstrated increased p38 activation and IL-6 production, corresponding to worsened hepatic damage and pulmonary inflammation. Furthermore, antecedent depletion of Kupffer cells or p38 inhibition after injury attenuated damage in the liver and lungs to the degree observed in post burn injuries without alcohol exposure (Figures 2 & 4, [19,89,111]). We recently reported near identical hepatic and pulmonary protection from limiting bacterial translocation after intoxication and burn as the current study where the ability of Kupffer cells to respond to LPS was impaired. Taken together, these results may suggest that intoxication worsens post burn outcome through sensitizing Kupffer cells while increasing the delivery of LPS to the liver. Of clinical importance is the finding that alcohol mediates this post burn response through the action of Kupffer cell p38 MAPK, perhaps revealing a much needed therapeutic target for this at risk patient population.
CHAPTER 5
ALCOHOL POTENTIATES POST BURN REMOTE ORGAN DAMAGE THROUGH
SHIFTS IN FLUID COMPARTMENTS MEDIATED BY BRADYKININ

Abstract

Of the 450,000 burn patients each year, 50% have a positive blood alcohol content and this predisposes them to worsened clinical outcomes. Despite high prevalence and established consequences, the mechanisms responsible for alcohol-mediated complications of post burn remote organ damage are currently unknown. To this end, mice received a single dose of alcohol (1.12 g/kg) or water by oral gavage and were subjected to a 15% total body surface area burn. Animals with a burn alone lost ~5% of their body weight in 24 hours whereas intoxicated and burned mice lost only 1% body weight (p<0.05) despite a 17% increase in hematocrit (p<0.05) and a 57% increase in serum creatinine (p<0.05) over burn injury alone. This retention of water weight despite increased dehydration suggests that intoxication at the time of a burn causes a shift in fluid compartments that may exacerbate end organ ischemia and damage as evidenced by a 3-fold increase in intestinal bacterial translocation (p<0.05), a 30% increase (p<0.05) in liver weight to body weight ratio, and an increase in alveolar wall thickness over a burn alone. Furthermore, administration of the bradykinin antagonist HOE140 30 minutes after intoxication and burn restored fluid balance and alleviated end
organ damage. These findings suggest that alcohol potentiates post burn remote organ damage through shifts in fluid compartments mediated by bradykinin.

**Introduction**

Remote organ damage after burns can be caused by aberrations in the inflammatory response, known as the systemic inflammatory response syndrome (SIRS), though the exact mechanisms are currently unknown. The physiologic response to a burn is dependent on the age of the patient, the size and severity of the injury, and patient-specific risk factors such as alcohol use. Alcohol is the most abused substance in the US and the third leading cause of preventable death [1], much of which is associated with unintentional injuries [120]. Binge drinking in particular is an increasingly prevalent form of intoxication [4] and is the characteristic pattern of drinking among patients presenting with traumatic injury, including burns [5]. Nearly 50% of patients admitted for burns have a positive blood alcohol content (BAC) at the time of admission and this predisposes them to worsened clinical outcomes compared to patients with similar injuries not under the influence of alcohol [121]. Specifically, intoxicated patients were found to be twice as likely to acquire an infection, required 60% more surgical procedures, had longer durations of stay in the intensive care unit, and generated more cost than their non-intoxicated counterparts [6,9]. With nearly 450,000 burns requiring medical attention each year in the American healthcare system [11], alcohol greatly contributes to the socioeconomic burden of this destructive injury as both a causative agent and complicating factor in recovery. Despite the high prevalence and established consequences of being intoxicated at the time of a burn injury, there are currently few
differences in the treatment and management of burn patients with and without prior alcohol exposure. This may be due in part to the dynamic natural history of burns as well as the complex and duration dependent effects of alcohol. In order to develop much needed targeted therapies, the effects of intoxication on the physiologic response to burn injury need to be studied and manipulated under controlled conditions.

Animal models have provided meaningful insight into the pathophysiology of burn injuries preceded by intoxication. Previous studies in our laboratory and others have demonstrated that relative to either insult alone, the combined injury of intoxication and burn results in elevated levels of neutrophil infiltration and edema in the lung [61], which occurs in an interleukin-6 (IL-6) dependent manner [89]. Furthermore, this pulmonary inflammation is accompanied by an increased susceptibility to pulmonary bacterial infections and decreased oxygen saturation compared to non-intoxicated controls [122]. This correlates with clinical data linking elevated serum IL-6 in trauma patients to mortality risk [123] as well as findings that burn patients with a positive BAC acquire more pulmonary infections [124]. Despite this knowledge, there remains a need to address the currently unknown mechanism by which alcohol intoxication worsens the post burn response and outcome. The need for such understanding is highlighted by the failure of single cytokine therapies in animal and clinical studies of similar injuries leading to SIRS [125]. Clinical data demonstrates intoxicated burn patients require more aggressive fluid resuscitation than their non-intoxicated counterparts [6] which may indicate some degree of hypovolemia. This is of significant concern as early ischemia-reperfusion injury may be the inciting event that initiates SIRS, which is linked to
pulmonary failure and ARDS [126]. One mechanism by which systemic capillary leakiness in this setting may ensue is through the vasoactive mediator, bradykinin. No one to date has investigated the role of bradykinin signaling in the setting of intoxication and burn but alcohol is known to potentiate the action of bradykinin [127] while burn injuries cause systemic bradykinin production [26]. To this end, the studies outlined herein examined a possible mechanism by which alcohol exacerbates post burn remote organ damage through potentiating third spacing of fluid and enhancing ischemic damage.

**Materials and Methods**

**Animals**

Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and sacrificed when 8-10 weeks old. Mice were housed in sterile micro-isolator cages under specific pathogen-free conditions in the Loyola University Chicago Comparative Medicine Facility. All experiments were conducted with approval of and strict accordance to the Loyola Institutional Animal Care and Use Committee.

**Murine Model of Intoxication and Burn Injury**

A murine model of a single binge ethanol exposure and burn injury was employed as described previously [107] with minor modifications. Briefly, mice were given a single dose of ethanol (1.12 g/kg) by oral gavage that results in a blood alcohol concentration of approximately 180 mg/dl at 30 minutes [19]. The mice were then anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), their dorsum shaved, weighed, and placed in a plastic template exposing 15% of the total body surface area and subjected to a scald
injury in a 92°C water bath or a sham injury in room-temperature water. The scald injury resulted in an insensate, full-thickness burn [108]. The mice were then resuscitated with 1.0 ml of 0.9% normal saline and placed on warming pads until recovered from anesthesia. At 30 minutes after burn injury, a separate group of mice was given the selective bradykinin receptor antagonist HOE 140 or saline control. HOE 140 stock solution (0.083 mg/mL) was prepared in saline and administered as a 150 µL intraperitoneal injection to achieve a dose of approximately 0.5 mg/kg. Mice were sacrificed by carbon dioxide narcosis followed by exsanguination 24 hours after injury. All experiments were performed a minimum of three times with one representative data set shown.

Blood and Serum Measurements

Blood was collected via cardiac puncture and an aliquot was placed into a microcapillary tube then measured for hematocrit by Hemavet (Drew Scientific, Dallas, TX). The remaining blood was harvested for serum by centrifugation after clotting. Serum aliquots were used to measure blood urea nitrogen and creatinine levels using a DRI-CHEM 7000 (HESKA, Loveland, CO).

Bacterial Translocation

Bacterial translocation was assessed as previously described [29,48]. Briefly, 3–5 mesenteric lymph nodes (MLN) per mouse were removed, placed in cold RPMI, and kept on ice. MLN were separated from connective tissue and mechanically homogenized in RPMI using frosted glass slides. Homogenates were plated in triplicate on tryptic soy
agar and placed in a 37°C incubator overnight. Colonies were counted the following day, averaged, and divided by the total number of lymph nodes harvested.

Histopathologic Examination of the Liver and Lungs

The whole liver was removed at the time of sacrifice, weighed, and normalized to the total body weight. The upper right lobe of the lung was inflated with 10% formalin and fixed overnight as described previously [110]. The lung was then embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Representative images were taken at 400x magnification.

Statistical Analysis

Statistical comparisons (GraphPad Instat) were made between the sham vehicle, sham ethanol, burn vehicle, and burn ethanol treatment groups, resulting in four total groups analyzed. One-way analysis of variance (ANOVA) was used to determine differences between treatment responses, and Tukey's post hoc test once significance was achieved (p<0.05). Statistical comparisons made between the intoxicated burned mice with saline or HOE140 treatment were performed using a Student's t-test and Tukey's post hoc test (p<0.05). Data are reported as mean values ± the standard error of the mean (SEM).

Results

Intoxication Increases Post Burn Dehydration Despite Fluid Retention

Sham injured animals with and without intoxication lost approximately 6% of their total body weight 24 hours after injury, presumably due to effects of anesthesia and
Figure 13. Fluid status 24 hours after injury. (A) Total body weight loss; (B) hematocrit, (C) creatinine, and (D) blood urea nitrogen (BUN) levels. *p<0.05 compared to all groups, #p<0.05 compared to sham groups by one-way ANOVA. N=4-6 animals per group.

stress of physical manipulation (Figure 13A). Similarly, mice receiving a burn alone lost >5% weight. When intoxication preceded the burn injury, however, mice lost 78% (p<0.05) less body weight than sham injured animals and 73% (p<0.05) less than mice
receiving a burn alone (Figure 13A). Despite this apparent retention of water weight, mice undergoing the combined injury of intoxication and burn had an approximate 30% increase (p<0.05) in hematocrit compared to sham injured animals regardless of alcohol exposure and a 17% increase (p<0.05) over a burn alone (Figure 13B). Similarly, intoxication and burn raised serum creatinine levels 3-fold (p<0.05) over sham injury and 1.5-fold (p<0.05) over burn alone (Figure 13C). A 2.3-fold increase (p<0.05) in serum blood urea nitrogen (BUN) was found after a burn alone compared to sham injury which was raised an additional 1.3-fold when preceded by intoxication (Figure 13D).

Remote Organ Damage is Increased when Intoxication Precedes Burn

Bacterial translocation to the mesenteric lymph nodes was increased >15-fold (p<0.05) after a burn injury compared to sham injured animals with and without alcohol exposure, and increased an additional 3-fold (p<0.05) when ethanol preceded the burn (Figure 14A). The increase in bacterial translocation with the combined injury was accompanied by a 40% (p<0.05) and 30% (p<0.05) increase in the relative size of the liver compared to sham injury and burn alone, respectively (Figure 14B). Histologic images of the lungs 24 hours after injury demonstrate an increase in alveolar wall thickness and cellularity with a burn alone which is exacerbated with antecedent intoxication (Figure 14C).
Figure 14. Remote organ damage 24 hours after injury. (A) bacterial translocation reported as the number of colony forming units (CFU) per mesenteric lymph node, (B) liver weight to total body weight ratio, (C) Lung histology by H&E staining. Representative images shown at 400x magnification. *p<0.05 compared to all groups by one-way ANOVA. N=4-6 animals per group.

Bradykinin Antagonism Partially Restores Fluid Balance after Intoxication and Burn

HOE140 treatment 30 minutes after intoxication and burn partially normalized weight loss at 24 hours injury as evidenced by a 10-fold (p<0.05) increase in weight loss compared to mice given saline vehicle (Figure 3A). Bradykinin antagonism with
HOE140 also alleviated dehydration after the combined injury as seen by a 12% decrease (p<0.05) in hematocrit (Figure 3B), 20% reduction (p<0.05) in serum creatinine (Figure 3C) and 20% lower (p<0.05) BUN (Figure 3D).

Figure 15. HOE140 restores fluid balance 24 hours after intoxication and burn. (A) Total body weight loss; (B) hematocrit, (C) creatinine, and (D) blood urea nitrogen (BUN) levels. *p<0.05 by Student’s T test. N=4-6 animals per group.
Bradykinin Antagonism Attenuates End Organ Damage after Intoxication and Burn

Early treatment with HOE140 after intoxication and burn injury led to a 47% reduction (p<0.05) in bacterial translocation to the mesenteric lymph nodes (Figure 4A), a 20% decrease (p<0.05) in liver weight to body weight ratio (Figure 4B) and attenuated pulmonary congestion as seen by a diminution of alveolar wall thickness and cellularity (Figure 4C).

Figure 16. HOE140 attenuates remote organ damage 24 hours after intoxication and burn. (A) Bacterial translocation reported as the number of colony forming units (CFU) per mesenteric lymph node, (B) liver weight to total body weight ratio, (C) lung histology by H&E staining. Representative images shown at 400x magnification. *p<0.05 by Student’s T test. N=4-6 animals per group.
Summary

The findings of this study suggest that alcohol potentiates post burn third spacing of fluid through the action of bradykinin, perhaps leading to greater intestinal ischemia and initial injury itself. We chose to examine a 24 hour time point because this allows for the greatest differences between treatment groups and avoids any confounding factors related to circadian rhythms. However, we have shown that there is increased intestinal damage as soon as 2 hours after injury when intoxication precedes burn [29]. Furthermore, because bradykinin antagonism partially restored fluid balance and attenuated intestinal damage we feel it can be concluded that bradykinin plays an important early role in this scenario, though more direct evidence of bradykinin involvement would be needed to be certain. This does not rule out the significance of other vasoactive agents after burn, such as histamine and serotonin, but given the potentiating effect of ethanol on bradykinin, B₂ antagonism may be particularly suitable therapeutic target in this combined injury.
INTOXICATION BY INTRAPERITONEAL INJECTION OR ORAL GAVAGE EQUALLY POTENTIATE POST BURN ORGAN DAMAGE AND INFLAMMATION

Abstract

The increasing prevalence of binge drinking and its association with trauma necessitate accurate animal models to examine the impact of intoxication on the response and outcome to injuries such as burn. While much research has focused on the effect of alcohol dose and duration on the subsequent inflammatory parameters following burn, little evidence exists on the effect of the route of alcohol administration. We examined the degree to which intoxication before burn injury causes systemic inflammation when ethanol is given by intraperitoneal (i.p.) injection or oral gavage. We found that intoxication potentiates post burn damage in the ileum, liver and lungs of mice to an equivalent extent when either ethanol administration route is used. We also found a similar hematologic response and levels of circulating interleukin-6 (IL-6) when either ethanol paradigm achieved intoxication before burn. Furthermore, both i.p. and gavage resulted in similar blood alcohol concentrations at all time points tested. Overall, our data show an equal inflammatory response to burn injury when intoxication is achieved by either i.p. injection or oral gavage, suggesting findings from studies using either ethanol paradigm are directly comparable.
Introduction

Ethanol is the most commonly abused substance in the United States and the third leading cause of preventable death [128], many of which are associated with unintentional injuries [2]. Binge drinking, defined as reaching a blood alcohol content of 0.08 [129], in particular, is an increasingly prevalent form of intoxication[4] and the characteristic drinking pattern of trauma patients [5]. As a central nervous system depressant, alcohol likely plays a causative role in many accidents but the diverse cellular effects of alcohol and its metabolites can also negatively alter the physiologic response to the injuries it helped cause [130]. As a small neutral compound capable of freely traversing lipid membranes, alcohol can influence nearly every cell in the body with effects dependent on the amount and duration of exposure [131]. Even a single dose of alcohol in animals has been shown to worsen systemic inflammation after injuries, such as burns [21,111]. Burns are a devastating injury with a complex natural history and high association with alcohol [132]. Nearly half of adult burn patients have a positive blood alcohol concentration (BAC) at the time of admission and this predisposes them to worsened clinical outcomes compared to patients with similar injuries not under the influence [120]. Specifically intoxicated patients were found to be twice as likely to acquire an infection, required more surgical procedures, had longer durations of stay in the intensive care unit and generated more cost than their non-intoxicated counterparts [6]. Interestingly, these patients are not typically chronic alcoholics but considered binge drinkers [133], consistent with the majority of alcohol consumption in the US [4]. With nearly 450,000 burns requiring medical attention each year in the American healthcare
system [11], alcohol greatly contributes to the socioeconomic burden of this destructive injury as both a causative agent and complicating factor in recovery. Despite the high prevalence and established consequences of binge intoxication at the time of burn injury, there are currently few differences in the treatment and management of burn patients with and without prior alcohol exposure. This may be due in part to the aforementioned dynamic natural history of burns as well as the complex and duration dependent effects of alcohol. In order to develop much needed targeted therapies, the effects of intoxication on the physiologic response to burn injury need to be studied and manipulated under controlled conditions. To this end, mouse models of binge ethanol exposure and burn have been in use for nearly 20 years and yielded insightful information into the mechanisms by which ethanol exacerbates the response to burn. Of note is the finding that ethanol can potentiate burn-induced damage in the intestine [33,44], liver [22,23] and lungs [61,134] with increased serum interleukin-6 playing an important role in inflammation of these organs [48,89]. The majority of these studies administer ethanol by gavage or i.p. injection to reach a desired BAC in mice. While presumably the presence and level of intoxication are the most important factors in these models, no one to date has investigated the impact of the route of ethanol administration in the context of burn. It is important to establish if results from historical experiments using i.p. injection and gavage are directly comparable as well as to be aware of any unintentional confounding factors in future studies. Herein we examine the effects of ethanol, given by gavage or i.p. injection, on post-burn inflammation and damage in the intestines, liver and lungs of mice.
Materials and Methods

Animals

Male wild-type (C57BL/6) mice were purchased from Jackson Laboratories (Bar Harbor, ME) and sacrificed at 8-10 weeks old. Mice were housed in sterile micro-isolator cages under specific pathogen-free conditions in the Loyola University Medical Center Comparative Medicine facility. All experiments were conducted in accordance with the Institutional Animal Care and Use Committee.

Murine Model of Ethanol and Burn Injury

A murine model of a single binge ethanol intoxication and burn injury was employed using either i.p. injection or oral gavage as described previously [107,135]. Briefly, i.p. mice were given a single i.p. dose of 150 μl of 20% (v/v) ethanol solution (1.12 g/kg) or saline control. Gavaged mice were given a single dose of 300 μl of 10% (v/v) ethanol solution (1.12 g/kg) or water control. The mice were then anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine), their dorsum shaved, placed in a plastic template exposing 15% of the total body surface area, and subjected to a scald injury in a 92-95°C water bath or a sham injury in room-temperature water. The scald injury results in an insensate, full-thickness burn [108]. The mice were then resuscitated with 1.0 ml saline and allowed to recover on warming pads. All experiments were performed between 8 and 9 am to avoid confounding factors related to circadian rhythms.

Blood Alcohol Concentration (BAC)

Mice were sacrificed at 30 minutes, 1 hour or 4 hours after a single dose of ethanol (1.12 g/kg) administered by either i.p. injection or gavage. Whole blood was
collected via cardiac puncture, incubated at room temperature for 20 minutes and then centrifuged at 3000 rpm at 4°C for 20 minutes. Serum was isolated and BAC was measured using the GM7 Micro-Stat Analyzer (Analox, Lunenburg, MA).

Blood and Serum Measurements

At 24 hours post-injury mice were euthanized, blood was collected via cardiac puncture, an aliquot was placed into a microcapillary tube, and read for a complete blood count with differential by Hemavet (Drew Scientific, Dallas, TX). The remaining blood was harvested for serum as described above and stored at -80°C. Serum aliquots were used to measure IL-6 by enzyme linked immunosorbent assay (ELISA) (BD Biosciences, Franklin Lakes, NJ) or liver transaminase levels using a DRI-CHEM 7000 (HESKA, Loveland, CO).

Histopathologic Examination of the Ileum and liver

At 24 hours post-injury mice were euthanized and the ileum, liver, and lungs were harvested. The ileum was fixed overnight in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). The length of 5 individual villi in 5 fields of view (100X) were measured for a total of 25 measurements per animal. The average was considered representative of the villus length in the ileum and demonstrative images are presented herein. The whole liver was removed at the time of sacrifice, weighed and normalized to total body weight.

Bacterial Translocation

Bacterial translocation was assessed as previously described [29]. Briefly, 3–5 mesenteric lymph nodes per mouse were removed and placed in cold RPMI, and kept on
ice. Nodes were separated from connective tissue and homogenized with frosted glass slides. Homogenates were plated on tryptic soy agar and incubated at 37°C overnight.

Histopathologic Examination of the Lung

The upper right lobe of the lung was inflated with 10% formalin and fixed overnight as described previously [110], embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Photographs were taken in a blinded fashion of 10 high power fields (400X) per animal and analyzed using the Java-based imaging program ImageJ (National Institutes of Health, Bethesda, MD). The images were converted to binary to differentiate lung tissue from air space and then analyzed for the percent area covered by lung tissue in each field of view as described previously [89]. Neutrophils were counted in a blinded fashion in 10 high power fields (400X).

KC Analysis of Lung Homogenates

The right middle lung lobe was snap-frozen in liquid nitrogen. The tissues were then homogenized in 1 ml of BioPlex cell lysis buffer according to manufacturer’s instructions (BioRad, Hercules, CA). The homogenates were filtered and analyzed for cytokine production using an ELISA for KC (BD Biosciences, Franklin Lakes, NJ). The results were normalized to total protein using the BioRad protein assay (BioRad, Hercules, CA).

Statistical Analysis

Statistical comparisons were made between i.p. and gavage animals in the sham vehicle, sham ethanol, burn vehicle, and burn ethanol treatment groups, resulting in 8 total groups analyzed. One-way analysis of variance was used to determine differences
between treatment responses, and Tukey’s post-hoc test once significance was achieved (p<0.05). Data are reported as mean values ± the standard error of the mean.

**Results**

**Blood Alcohol Concentration is Equal after I.P. Injection or Gavage**

To determine if the route of ethanol administration impacted the kinetics of its absorption and clearance, the blood alcohol concentration (BAC) of mice was determined at 30 minutes, 1 hour and 4 hours after a single dose of 1.12 g/kg ethanol by i.p. injection or gavage. Mice receiving ethanol by i.p. injection were found to have a BAC of 143 mg/dL at 30 minutes, which was reduced 33% by 1 hour and 69% by 4 hours (Figure 17). Similarly, mice receiving ethanol by gavage demonstrated a BAC of 141 mg/dL at 30 minutes, which by 1 hour was decreased by 41% and by 77% at 4 hours (Figure 17). No significant difference between BAC in mice receiving ethanol via i.p. injection or gavage at each time point was found, suggesting that equivalent amounts of ethanol absorb into the bloodstream and are cleared at similar rates.

**Intoxication by I.P. Injection or Gavage Increases Peripheral Blood Granulocytes after Burn**

To examine if administration route effected the hematologic response to intoxication and burn, the number of circulating granulocytes was enumerated by an automated counter after burn or sham injury when preceded by ethanol given by i.p. injection or gavage. In gavaged mice, there was a 2-fold increase (p<0.05) in blood granulocytes in intoxicated burned mice relative to sham injured mice regardless of prior
Figure 17. Blood alcohol concentration (BAC). Mice were administered 1.12g/kg ethanol and the subsequent BAC measured at various time points. Data are presented at mean values ± SEM. N=6-9 animals per group.

intoxication status (Figure 18). Likewise in mice given an i.p. ethanol injection before burn, a 3-fold increase (p<0.05) in blood granulocytes was found when compared to sham injured mice with and without prior intoxication (Figure 18). No significant differences were found between i.p. injected and gavaged mice within treatment groups suggesting both routes of ethanol administration induce an equal neutrophilic leukocytosis after burn injury.

Serum IL-6 is Elevated When Intoxication Precedes Burn

Injury Regardless of Administration Route

Circulating IL-6 levels were quantified by ELISA in all treatments groups of i.p. injected and gavaged mice. Burn injury alone increased the amount of serum IL-6 by greater than 25-fold above sham injured animals in both i.p. and gavage mice (Figure 19). When intoxication preceded the burn, a further 3 to 4-fold increase (p<0.05) above burn
Figure 18. Circulating blood granulocytes 24 hours after injury. *p<0.05 compared to Sham groups. Data are presented at mean values ± SEM. N=3-6 animals per group.

Figure 19. Serum IL-6 at 24 hours after injury. *p<0.05 compared to Sham groups. Data are presented at mean values ± SEM. N=4-8 animals per group.
alone was observed, regardless of the route of ethanol administration (Figure 19).

No significant differences were found between i.p. and gavage mice within treatment groups suggesting that intoxication before burn injury increases serum IL-6 irrespective of the ethanol paradigm used.

Villus Blunting is Similar in I.P. and Gavage

Intoxicated Mice After Burn

We previously reported that intoxication by i.p. injection furthers the diminution of ileal villi after burn [29]. Consistent with our earlier observations, at 24 hours after burn (Figure 20, E-F), villi in the ileum were shortened in comparison to sham injured animals regardless of intoxication status (Figure 20, A-D). Furthermore when mice were intoxicated by i.p. injection (Figure 20, G) or gavage (Figure 20 H), villus blunting was pronounced beyond burn alone.

The average villus length in the ileum of burn injured mice was blunted by greater than 20% (p<0.05) compared to sham injured animals regardless of intoxication status or administration method (Figure 21A). Antecedent intoxication by i.p. injection or gavage caused a further ~20% reduction (p<0.05) compared to burn alone (Figure 21A), demonstrating this increased intestinal damage is present to a similar extent whether intoxication is achieved by i.p. injection or gavage. This villus blunting corresponded to an increase in bacterial translocation to the mesenteric lymph nodes where intoxication increased the number of colony forming units by >400-fold over sham animals and 5-fold over burn alone (Figure 21B). No significant differences between i.p. and gavage mice
were found within treatment groups suggesting both routes of ethanol administration
effect intestinal damage after burn injury in a similar manner.

Figure 20. Histologic state of the ileum 24 hours after intoxication and burn injury. The
first row is representative of villi in the ileum of sham injured mice given vehicle by i.p.
injection (A) or gavage (B). The second row show villi from sham injured mice given
ethanol by i.p. injection (C) or gavage (D). The third row villi appear rounded and
widened as seen in burned mice given vehicle by i.p. injection (E) or gavage (F). The
final row demonstrate the villus blunting observed in burn injured mice administered
ethanol by i.p. injection (G) or gavage (H).
I.P. or Gavage Intoxication Equally Exacerbates Hepatic Damage After Burn

Hepatic damage was measured by levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and by the liver weight to total body weight ratio. Burn alone increased serum ALT levels by greater than 6-fold compared to sham injured animals (Figure 22A). This increase was found irrespective of the presence or absence of ethanol or route of administration before sham injury. When i.p. or gavage mice were intoxicated at the time of burn however, a greater than 12-fold elevation (p<0.05) was observed over sham injured animals which corresponded to a ~2-fold increase (p<0.05) above burn alone (Figure 22A). A similar pattern was found for serum AST with burn alone causing a greater than 5-fold increase relative to sham injured groups in both i.p. injected and gavaged mice with and without ethanol (Figure 22B).
Intoxication in both i.p. and gavage mice at the time of burn increased serum AST an additional 2-fold (p<0.05) which is a greater than 20-fold elevation (p<0.05) over sham injured mice (Figure 22B).

Figure 22. Hepatic damage 24 hours after injury. Serum alanine aminotransferase (ALT) (A) and serum aspartate aminotransferase (AST) (B) 24 hours after injury. *p<0.05 compared to Sham and Burn Vehicle groups. Data are presented at mean values ± SEM. N=3-5 animals per group.

Finally, the liver weight to total body weight ratio (LW:BW) was recorded as a measure of hepatic edema. No significant changes in LW:BW were found between sham groups regardless of ethanol intoxication or administration route (Figure 23). Similarly, burn alone did not cause a significant change in LW:BW relative to sham injured mice. However when mice received ethanol by i.p. injection or gavage before burn, a ~47% increase (p<0.05) above all other groups was observed. Taken together, the serum transaminase and LW:BW suggest that ethanol potentiates liver damage after burn injury irrespective of the route intoxication was achieved.
Figure 23. Liver weight to body weight ratio 24 hours after injury. *p<0.05 compared to Sham and Burn Vehicle groups. Data are presented at mean values ± SEM. N=4-6 animals per group.

I.P. or Gavage Administration of Ethanol Enhances Alveolar Wall Thickness After Burn

At 24 hours after intoxication and burn injury, there is a marked increase in the thickness of the alveolar wall and increased cellularity, which is more pronounced than after burn alone (Figures 24 and 25). The alveolar wall thickness and cellularity was quantified using imaging software to measure the area of lung tissue in 10 high power fields per animal which is reported as a percentage of the entire field of view. A significant increase in tissue area, corresponding to a relative decrease in air space, was found after burn injury, compared to sham animals (p<0.05). Intoxication increased the tissue area after burn regardless of how it was achieved (p<0.05), indicating a greater level of pulmonary congestion.
Figure 24. Histologic state of the lungs 24 hours after injury. The first row is representative of the lungs of sham injured mice given vehicle by i.p. injection (A) or gavage (B). The second row show lungs from sham injured mice given ethanol by i.p. injection (C) or gavage (D). The third row displays an increase in alveolar wall thickness as seen in burned mice given vehicle by i.p. injection (E) or gavage (F). The final row demonstrate the amplified alveolar wall thickness and cellularity in burn injured mice administered ethanol by i.p. injection (G) or gavage (H).
Neutrophil Accumulation and Pulmonary KC Levels are Amplified after I.P. or Gavage Intoxication and Burn

Similar to previous studies [61,110,134], following the combined insult of i.p. ethanol injection and burn, there was a 20-fold increase in pulmonary neutrophils compared to sham animals (p<0.05) and a 2-fold increase over burn alone (p<0.05) (Figure 26A). This neutrophil accumulation in i.p. intoxicated animals corresponded to a 6-fold increase in KC compared to sham animals (p<0.05) and a 2-fold elevation compared to burn alone (Figure 26B). When an equal amount of ethanol was given by gavage, similar results were observed with intoxicated burned mice having neutrophil numbers and KC levels that were 15 and 6-fold above sham animals (p<0.05) and 2.5 and 2-fold above burn alone, respectively (Figure 26). No significant differences between i.p. and gavage mice within treatment groups were found suggesting that intoxication enhances post-burn pulmonary neutrophil accumulation and KC regardless of administration route.

Figure 25. Quantification of pulmonary congestion 24 hours after injury. *p<0.05 compared to Sham and Burn Vehicle groups. #p<0.05 compared to Sham groups. Data are presented at mean values ± SEM. N=4-6 animals per group.
Figure 26. Pulmonary inflammation after intoxication and burn. Pulmonary neutrophils in 10 high power (400x) fields of view (A) and pulmonary KC levels (B) 24 hours after injury. #p<0.05 compared to Sham groups. *p<0.05 compared to Burn Vehicle groups. Data are presented at mean values ± SEM. N=4-8 animals per group.

Summary

Animal studies using mice offer controlled conditions, manipulatable genomes, and pharmacologic interventions not available in humans. An important variable is the level of intoxication achieved before burn and while historically animal studies have administered known amounts of ethanol by i.p. injection, oral gavage is considered a more physiologic method of intoxication. We now describe that post burn inflammation and damage in the ileum, liver and lungs of mice are exacerbated to an equal extent when preceded by intoxication achieved by i.p. injection or gavage. Furthermore, the administration route had no impact on the hematologic changes observed when intoxication precedes burn. Taken together our data suggest that either i.p. injection or gavage are appropriate for studying the effects of ethanol on post burn inflammation and response.
CHAPTER 7
DISCUSSION

As a small neutral compound with the ability to cross lipid membranes, alcohol can affect nearly any cell in the body with varying consequences depending on the context, amount and duration of exposure [131]. Burns are a devastating and dynamic injury involving potential complications in every major organ system including cardiac impairment [136], renal dysfunction [137], intestinal damage [138], hepatic derangements [14] and pulmonary failure [139]. It is unlikely, given the permeating effects of both burns and alcohol, that there is a single cell type or molecule solely responsible for the multi-system sequelae observed in burn patients who were intoxicated at the time of their injury. Rather, the involvement of several organs, each influencing the response of others seems plausible and supported by findings reported herein.

The Role of the Intestinal Barrier

Emerging evidence points to a strong relationship between intestinal microbiota and the liver which is proposed to regulate a myriad of human diseases [49,50,51,52,53]. Independently, burn injury [23] and alcohol [27,53] have also been shown to alter aspects of the gut-liver axis but the combination of the two insults is relatively unexplored despite its prevalence in the trauma population [4,5]. We have previously reported that in mice, antecedent ethanol exposure potentiates post burn intestinal damage [19,29,48], hepatosteatosis [23,55] and IL-6 production [19,21,48,56], as well as exacerbates
pulmonary inflammation [19,61,89,134] compared to either intoxication or a burn alone. In Chapter 3, we demonstrate through the use of two separate approaches that gut-derived bacteria, or bacterial products, may play a causative yet reversible role in the well-established ability of ethanol to worsen post burn systemic inflammation.

The intestinal barrier is normally maintained by multiple types of junction complexes including the tight junction proteins ZO-1 and occludin. In our current study, we chose to investigate only ZO-1 localization as a representation of tight junction integrity but have verified similar results with occludin in this animal model elsewhere [96,140]. In agreement with these studies, PIK treatment restored intestinal barrier function after injury (Figure 4A) and we now report the benefits of this treatment on the hepatic and pulmonary responses to intoxication and burn. The finding that PIK treatment dampened steatosis development after intoxication and burn (Figure 4) suggests that the aberrant hepatic response observed in this setting is caused, at least in part, by the translocation of intestinal bacteria. Furthermore, decreasing intestinal bacterial load before injury alleviated these parameters to an equal extent. These findings do not rule out the possible influence of other etiologies of steatosis (see Figure 3), such as oxidative stress [141] or changes in fat metabolism [14] but are supported by data from autopsies of burn patients which reveal an association between fatty liver infiltration and bacterial translocation from the intestines [54]. Similarly, in a hemorrhagic shock model, gut sterilization of rats was found to confer hepatic protection [142].
The degree of steatosis in our intoxicated burned mice paralleled other markers of hepatic damage as measured by serum AST, ALT and relative size of the liver after injury (Figures 5A-C). Severe derangement of AST and ALT in burn patients are a harbinger for complications and death [15] and the liver can increase its size by 225% after a burn alone [143]. The liver is also the major site of ethanol metabolism and toxicity which is known to sensitize the liver to gut-derived LPS [74,95,96]. Ethanol priming of the liver to post burn intestinal LPS [73] may help explain our findings that intoxication potentiated post burn hepatic damage which was attenuated with either PIK or antibiotic treatment (Figure 5). The etiology of acute liver injury in the setting of intoxication and burn is of significant clinical concern as the liver is a central player in regulating the inflammatory response that is thought to be responsible for the worsened prognosis [144].

An important player in any inflammatory response, IL-6 is particularly elevated in thermally injured patients [84,85] and correlates to increased mortality risk after injury [86]. Moreover we and others have reported that antecedent intoxication significantly raises IL-6 levels of burn injured mice [19,21,23] and reducing IL-6 attenuates pulmonary inflammation [89] and gut permeability [48], possibly serving as a link between the gut, liver and lung in this common clinical scenario. The studies of Chapter 3 demonstrate that hepatic IL-6 production at the mRNA and protein level can be manipulated through limiting hepatic exposure to intestinal bacteria (Figure 6A&B). Furthermore, this correlates to IL-6 levels in the serum (Figure 6C) indicating the liver
could be the source of systemic IL-6 when burn is preceded by ethanol exposure. This agrees with studies of other trauma models where the liver is considered the source of systemic IL-6 after injury [88]. Additionally, Li et al [21] found elevated IL-6 in the plasma, ileum, liver and lungs of mice receiving ethanol and burn injury with the highest concentration of IL-6 in the liver.

As discussed in Chapter 2, IL-6 production after intoxication and burn in the liver may be initiated through the interaction between gut-derived LPS and TLR4 on Kupffer cells. Residing in the sinusoids of the liver, Kupffer cells are tissue-fixed macrophages that survey the blood for circulating antigen and mobilize an immune response. After burn there is an increase in circulating LPS [27] which, through binding to TLR4, stimulates Kupffer cells to express inflammatory genes and produce cytokines such as IL-6 [60]. Ethanol exposure alters TLR4 signaling in a variety of ways including changes to CD14 expression [73], lipid rafts [145], and mitogen-activated protein kinases (MAPKs)[146,147], which can increase IL-6 production after exposure to LPS (See Figure 2). Furthermore, the absence of TLR4 in the setting of intoxication and burn significantly decreases circulating IL-6 [61]. The experiments of Chapter 4 investigate the role of Kupffer cells in this setting but evidence from other models of trauma support the idea that Kupffer cells may be the systemic source of IL-6 after injury [88].

Increased serum IL-6 is linked to poor survival in patients with acute respiratory distress syndrome (ARDS) [148], though whether IL-6 plays a causative role or is simply a marker of mortality risk remains to be conclusively demonstrated. Likewise, alcohol
abuse is also associated with a worsened ARDS prognosis [149] and is a common complication after a thermal injury [150]. ARDS is characterized by inflammation and edema in the lung parenchyma leading to impaired gas exchange. Similarly, Figure 7A demonstrates an increase in alveolar wall thickness in the combined injury and is consistent with previously reported results [19,61,89,122,134]. The degree of reduction in pulmonary inflammation observed with PIK treatment (Figure 7) matches the level of reduction found in previous studies of IL-6 deficient mice undergoing the same combined injury [89], suggesting the pulmonary benefit in PIK-treated animals may be related to decreased IL-6 levels. The parallel results in antibiotic-treated mice imply the pulmonary benefits of PIK treatment stem from the limitation of bacterial translocation and not a direct action of PIK on the lungs.

**Kupffer Cell Regulation of the Hepatic Response**

After a severe burn injury, hepatic dysfunction and associated hypermetabolism can persist for years despite clinical intervention [24]. Animal studies suggest the occurrence of hepatic derangement is dependent on the size of the burn [18]. In a direct comparison, substantial hepatic derangements were observed in a 40% TBSA burn that were absent when reduced to 20% [18]. The authors concluded from this work that increasing the severity of injury does not lead to a simple dose-dependent response but rather qualitatively different responses, though this may also suggest an injury threshold beyond which an aberrant hepatic response occurs. Work from our lab and others demonstrate significant hepatic damage in a mouse model of 15% TBSA burn only when
intoxication precedes the injury \[19,20,21,22,23\]. This may indicate that alcohol lowers the threshold for an aberrant post burn hepatic response. Many overlapping physiologic effects of both alcohol and burn converge on Kupffer cell activity (See Figure 2) that may work in a synergistic fashion to lower this threshold for hepatic damage and derangement, as supported by the findings contained herein.

We demonstrate that a single intoxication event increases post burn sensitivity of Kupffer cells to LPS to a greater extent than after a burn alone (Figure 8). This is of clinical importance as Kupffer cell hyperactivity is known to cause hepatic damage after many types of injury including hemorrhagic pancreatitis [65], trauma and sepsis [66], as well as burns [67]. Kupffer cell sensitization to LPS has been shown to occur in a rat models of 30% TBSA burn [67,113], which agrees with the results of Kupffer cells isolated from burn-injured animals in Figure 8 and is supported by work with TLR-4 defective animals [69]. Additionally, alcohol, even after a single dose, also increases Kupffer cell sensitivity to LPS [73], a finding recapitulated in Figure 8 and supported by work demonstrating the role of Kupffer cells in mediating alcohol-induced liver damage [151]. While burn injury or alcohol may sensitize Kupffer cells via a variety of mechanisms including changes in CD14 expression [73], lipid rafts [145], and mitogen-activated protein kinases (MAPKs)[146,147], the functional consequence is increased TLR4 signaling and cytokine production. Accordingly, we have previously demonstrated that the absence of TLR4 attenuates systemic IL-6 and remote organ damage 24 hours after intoxication and burn [61]. However, animal models of systemic inflammation
suggest overall outcome may require a balance of maintaining Kupffer cell function without over-stimulation [64], highlighting the need to understand the underlying mechanisms of Kupffer cell hyperactivity. Of the many downstream signaling proteins of TLR4, p38 MAPK may have particular importance in the setting of intoxication and burn.

A member of the MAPK family, p38 has a well-established role in post burn remote organ damage [75,76,77,78,79], is altered by alcohol exposure [80] and enhances TLR4 reactivity following injury [82]. We found increased p38 activity in Kupffer cells isolated from burn-injured animals (Figure 8A), a finding that has also been reported after trauma-hemorrhage [152]. In the combined injury, significantly increased Kupffer cell p38 activation (Figure 8A) and IL-6 production (Figure 8B) was found over either insult alone, mirroring the degree of damage at the organ level (Figures 9&11). These observations from isolated Kupffer cells demonstrate that ethanol augments p38 activation in Kupffer cells after burn, which corresponds to heightened IL-6 production. Furthermore, *ex vivo* inhibition of p38 (Figure 8A) abrogated LPS-induced IL-6 production (Figure 8B) suggesting that the increased IL-6 after intoxication and burn is produced in a p38-dependent manner.

In order to further examine the role of Kupffer cell p38 in the hepatic response to burns preceded by alcohol exposure, mice were antecedently depleted of Kupffer cells with clodronate liposomes or given a p38 inhibitor after injury. The clodronate dose and administration route used is a well-established method to achieve relatively selective
depletion of Kupffer cells ([114], Figures 12A&B), while leaving other macrophage populations such as the circulating monocytes (Figure 12C) and alveolar macrophages (Figures 12D&E) intact. The dosage of p38i used has previously been shown to inhibit p38 activation in Kupffer cells 24 hours after burn [113] which we confirm through decreased p38 phosphorylation (Figure 12F) and IL-6 production (Figure 12G) after LPS stimulation.

Similar to the results of Chapter 3, the absence of Kupffer cells conferred hepatic protection from intoxication and burn as measured by decreases in serum AST, serum ALT, and the liver to body weight ratio. It is interesting therefore that global p38 inhibition attenuated hepatic damage to an extent equal to that of antecedent Kupffer cell depletion, suggesting that p38 specifically within Kupffer cells may be responsible for the increased hepatic damage. Furthermore, both treatments reduced hepatic damage to the extent seen after a burn alone, implying that alcohol may potentiate post burn hepatic damage through Kupffer cell p38 activity. Similarly, steatosis was reduced with either treatment to the degree of a burn alone, implying a prominent role for Kupffer cell p38 in hepatic triglyceride accumulation after intoxication and burn. While these findings do not rule out the possible influence of other etiologies of steatosis (see Figure 3), they are supported by recent evidence which indicates Kupffer cells can promote steatosis via increased cytokine production [71].

Chapter 3 demonstrated that the increased hepatic IL-6 production when intoxication precedes burn is dependent on gut-derived LPS. The present studies
demonstrate that hepatic IL-6 production can also be manipulated through p38 inhibition or Kupffer cell depletion (Figure 10). This may be important as IL-6 at low levels is beneficial to hepatocyte survival but above a threshold of excessive amount or prolonged duration can be detrimental [62]. IL-6 levels in the serum (Figure 10B) paralleled hepatic IL-6 reinforcing that the liver could be the source of systemic IL-6 when burn is preceded by ethanol exposure.

The degree of reduction in pulmonary inflammation observed with p38i treatment (Figure 11) matches the level of reduction found in previous studies of IL-6 deficient mice undergoing the same combined injury [89], suggesting the pulmonary benefit in p38i-treated animals may be related to decreased IL-6 levels. This matches the studies of Chapter 3 where decreased IL-6 attenuated neutrophil accumulation in the lung (Figures 6&7). The parallel results in clodronate-treated mice imply the pulmonary benefits of p38i treatment stem from its effect on Kupffer cells and not a direct action of p38 inhibition in the lungs. While more studies would be needed to confirm the precise action of Kupffer cell p38 on pulmonary inflammation after intoxication and burn, our findings are supported by work demonstrating a direct role of Kupffer cells in lung injury during endotoxic shock [153].

**The Influence of Fluid Shifts**

Chapters 3 and 4 demonstrate that the gut-liver axis is paramount to the post burn response and can be manipulated through blocking the interaction of gut-derived LPS and Kupffer cells after injury. With this in mind we sought to examine possible causes for
the increased intestinal damage and permeability when intoxication precedes burn. Full-thickness burns can induce excessive vascular permeability both at the site of injury and in remote organs, leading to detrimental hypodynamic changes in circulation [26]. Burn patients who were intoxicated at the time of their injury require greater amounts of fluid resuscitation [6], perhaps indicating a greater degree of fluid extravasation. This is supported by findings contained in Chapter 5 where mice given ethanol and burn injury have increased hematocrit, creatinine, and BUN compared to mice given either ethanol or burn alone (Figures 13B-D). Interestingly, the combination of intoxication and burn led to weight retention 24 hours after injury (Figure 13A). This apparent retention of fluid despite increased dehydration suggests that intoxication at the time of a burn causes a shift in fluid compartments that may exacerbate end organ ischemia and damage in the intestines (Figure 14A). Catecholamine-mediated vasoconstriction of the splanchnic vasculature makes the intestines particularly susceptible to hypovolemia [25], made worse by third spacing of fluid as evidenced by edema in the liver (Figure 14B) and lungs (Figure 14C). One mechanism by which systemic capillary leakiness in this setting may ensue is through the action of the vasoactive agent, bradykinin.

Bradykinin is a potent vasoactive agent produced systemically after a burn with effects of vasodilation and vascular permeability [26]. The effects of bradykinin are mediated by two cellular receptors classified as B₁ and B₂. B₁ receptors are present only at low levels in healthy tissues while B₂ receptors are constitutively expressed in most issues. Acute inflammatory responses such edema formation are mediated through B₂
receptors whereas chronic inflammation is associated with B1 activity [154]. Accordingly, most experimental and clinical studies examining bradykinin antagonism in an acute inflammatory setting have focused on B2 receptor blockade. HOE140 is a potent, selective, and long-acting B2 receptor antagonist and has been used to treat experimental sepsis [155], acute pancreatitis [156], and local inflammation [157] or microvascular changes [158] after thermal injury though with varying degrees of success. B2 receptor antagonism may be particularly suited to treat the common clinical scenario of intoxication and burn injury because burn injuries cause systemic bradykinin production [26], while its action is known to be potentiated in the presence of alcohol [127], perhaps affording bradykinin undue influence on vascular permeability after this combined injury. In agreement with this, we found that B2 blockade early after intoxication and burn restores fluid balance (Figure 15) and attenuates end organ damage (Figure 16). This highlights the early important role of bradykinin after burn with antecedent alcohol exposure and also suggests third spacing is at least partially responsible for the increased remote organ damage in this setting.

The pathophysiology of both binge intoxication and burn injury converge on bradykinin signaling and could explain numerous observations in animal models and patients as described above. The exacerbated injury in the intestines, liver, and lungs of mice when ethanol precedes burn injury is well documented and agrees with and expands upon the findings of Figure 14. Therapeutic interventions in animals to date have focused on mitigating events downstream of the initial injury, such as manipulating levels of IL-6.
[89], IL-18 [159], intracellular adhesion molecule-1 (ICAM-1) [160], and Toll-like receptor 4 (TLR4) [61]. While some of these interventions have proven efficacious in providing protection in various organs, as a whole they focus on restoring aberrations that follow the combination of intoxication and burn instead of addressing the underlying cause of the exacerbated injury.

**Ethanol Administration in our Animal Model**

In order to more accurately represent physiologic drinking in our animal model, the route of ethanol administration was changed from being given via i.p. injection (Chapter 3) to oral gavage (Chapters 4&5). The studies in Chapter 6 indicate that in the context of burn injury in mice, intoxication at an equivalent BAC and duration exacerbates organ inflammation and damage to a similar extent whether given by oral gavage or i.p. injection. Equal BAC’s at the time of injury resulting in comparable amounts of organ damage is consistent with findings that suggest ethanol acts through worsening ischemic damage [161], altering cytokine networks [162,163], and impairing immune responses [144] after burn injury and perhaps not through interactions at the site of absorption. The near infinite water solubility of ethanol allows for a quick distribution throughout the blood and we observed peak BACs near 30 minutes when ethanol was administered by oral gavage or i.p. injection. As seen in Figure 17, identical doses of ethanol by both paradigms resulted in nearly the same BAC at 30 minutes, 1 hour and 4 hours after administration. The absorption time and BAC of the i.p. mice agree with our previously published studies [23,29,163] but the equivalency of BAC profiles between
gavage and i.p. injection are in contrast with work by Livy et al [164] who concluded that in mice, ethanol given by gavage resulted in a lower BAC than an equivalent amount of ethanol given by i.p. injection. They proposed this discrepancy may be due to metabolism by gastric alcohol dehydrogenase, which only occurs when ethanol traverses the gastrointestinal tract. While the reasons for the contrasting results are unclear, several possibilities, including differences in ethanol amount (3.8g/kg vs 1.12g/kg), the volume of ethanol administered (up to 0.41 ml vs 0.15 ml (i.p.)), and discrepancy between the vehicle used in i.p. injections (water vs saline) may have been contributing factors. Nevertheless, the mice used in our studies, which were given equal doses of ethanol by gavage and i.p. injection, demonstrated equivalent BAC profiles and an over exuberant inflammatory response after burn. A further discussion regarding considerations of i.p. and gavage ethanol administration can be found elsewhere as reviewed by D’Souza El-Guindy et. al. [165].

A neutrophilic leukocytosis is seen in a variety of illnesses and conditions and is widely regarded an indicator of infection or inflammation. Trauma can also induce a leukocytosis where it is considered an acute phase marker and is clinically associated with increased morbidity and mortality risk [166]. We observed, in Figure 18, that intoxication by either paradigm induced a similar granulocytic leukocytosis at 24 hours after burn. The sequestration of these circulating neutrophils in end organs after injury is proposed as a major mechanism in the pathogenesis of multiple organ failure [167]. We and others have shown that intoxication at the time of burn leads to increased neutrophil
infiltration into the gut, liver and lungs of mice within 24 hours [21,111,168]. Furthermore, prevention of neutrophil transmigration using ICAM knockout mice in this setting decreased pulmonary inflammation [134], highlighting the important role of neutrophil infiltration in this setting.

Circulating neutrophils migrate from the blood into tissues along a density gradient of chemoattractants, which in the mouse include KC. In mice, a burn injury increases pulmonary KC and ethanol has been shown to amplify this accumulation both in the absence [61,89,134] and presence of an intratracheal infection with *Pseudomonas aeruginosa* [122,163]. We observed that both ethanol paradigms increase pulmonary KC equally after burn (Figure 26B) and this corresponded to increased neutrophil numbers in the lung (Figure 26A). The leukocytosis after intoxication and burn, together with an increase in neutrophil chemoattractants, likely plays a key role in the subsequent pulmonary inflammation and appears to be independent of the method of ethanol administration.

As discussed above elevated levels of circulating IL-6 also correlate with mortality risk in trauma patients [169] and are further increased when intoxication precedes burn injury [23,56,131]. We confirm our previous findings that burn alone increases serum IL-6 levels in mice and intoxication at the time of injury raises circulating IL-6 even further. We now report that this amplified IL-6 level when intoxication precedes burn injury is not affected by the route of ethanol administration (Figure 19).
We report in Chapter 6 that gavage or i.p. intoxication potentiated post burn intestinal damage as demonstrated by histology (Figure 20) and villus length (Figure 21A). Furthermore, intestinal damage corresponded to an increase in bacterial translocation (Figure 21B) and hepatic damage as assessed by serum transaminase levels (Figure 22) and hepatic weight (Figure 23). These findings were independent of the ethanol administration route in our model and support the ability to directly compare results between studies using either administration route.

When examined by histology (Figure 24), the lungs of mice from both gavage and i.p. paradigms appear congested relative to all other treatment groups. When the amount of tissue relative to air space was quantified (Figure 25), the alveolar wall thickening and increased cellularity seen visually was found to be increased after burn and further increased with prior intoxication. This finding agrees with our previously reported work with i.p. injected mice [89] and is unaffected by the route of administration.

**Final Conclusions**

The increasing prevalence of binge drinking and its high association with traumatic injury warrant further investigation into the mechanisms underlying the worsened clinical outcomes associated with this combined injury. The existing literature reviewed in Chapter 2 suggests that the liver is at the center of the post burn systemic response with crosstalk between the intestinal microbiome and the liver playing an important role after injury. There is a paucity in the literature, however, regarding the role of the gut-liver axis in the common clinical scenario of burns preceded by alcohol
intoxication. Previous studies in our laboratory demonstrated intoxication potentiates post burn hepatic damage and IL-6 production in a dose-dependent manner. In Chapter 3 we show through two separate approaches that interruption of gut-liver crosstalk, through restoration of intestinal barrier function or prophylactic sterilization of the gut, attenuates hepatic damage and IL-6 production after the combined injury, highlighting the role of intestinal damage and subsequent bacterial translocation. In Chapter 4 we demonstrate, through antecedent Kupffer cell depletion, that Kupffer cells play a critical role in mediating the injurious hepatic response to intoxication and burn. Furthermore we show that alcohol increases post burn Kupffer cell sensitivity to LPS through increased p38 MAPK signaling, providing a potential mechanism for the increased hepatic and systemic levels of IL-6 found in the combined injury. Accordingly, global p38 inhibition also attenuated hepatic damage and IL-6 production in alcohol-exposed and burn injured

<table>
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<tr>
<th>Consequence of intoxication and burn</th>
<th>Experimental Intervention</th>
<th>Mechanism of Intervention</th>
<th>Post injury effect</th>
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<tr>
<td>Intestinal permeability</td>
<td>Restoration of tight junction complexes</td>
<td>↓ LPS delivery to liver</td>
<td>↓ Hepatic damage</td>
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<td>Antibiotics</td>
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<td>Hepatic damage</td>
<td>p38 inhibition</td>
<td>↓ Kupffer cell hyperactivity</td>
<td>↓ Pulmonary inflammation</td>
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<td>Depletion of Kupffer cells</td>
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<td>↓IL-6</td>
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<td>Fluid extravasation</td>
<td>Bradykinin antagonism</td>
<td>↓ intestinal damage</td>
<td></td>
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</tbody>
</table>

Table 1. Interventions attempted in the setting of intoxication and burn.
Taken together, the studies of Chapter 4 suggest that specifically targeting Kupffer cell p38 in the setting of intoxication and burn may have therapeutic benefit. This was supported by the decreased pulmonary inflammation associated with lowered IL-6 levels in all experimental interventions used (Table 1). In Chapter 5 we found that alcohol increased intestinal damage through potentiating bradykinin signaling, leading to greater fluid extravasation from the vascular compartment. The subsequent intestinal barrier breakdown may be the inciting event that alters the gut-liver axis observed in Chapter 3 (Figure 27).

Figure 27: Overlapping responses to burn injury and alcohol exposure alter the gut-liver axis. interleukin-6 (IL-6), Kupffer cells (KC), lipopolysaccharide (LPS), tumor necrosis factor (TNF)
In summation, the studies contained herein reveal that the gut-liver axis drives pulmonary inflammation after intoxication and burn injury. We demonstrate multiple mechanisms by which alcohol modulates the post burn hepatic response and provide preliminary evidence for potential therapeutic targets for this common clinical scenario.
Intoxication and Burn Injury Protocol

Materials

Hot plate
Water basins
Animal clippers with #40 surgical clip comb
Thermometer
1cc syringes with 27 gauge needle
3cc syringes with 20 gauge oral gavage needle
0.9% normal saline (sterile)
Burn injury template (check for proper size according to weight of mice)
Ketamine
Xylazine
Ethanol

Procedure

1. Fill two water basins with deionized water and heat one until 92-95°C, leaving the other at room temperature.
2. Tail mark and weigh the mice.
3. Gavage mice with ethanol at the desired dose with 150uL of ethanol mixed in water or water alone. Allow 2-3 minutes between cages so there is time to shave and burn the mice at their scheduled time.
4. Thirty (30) min. after ethanol administration, anesthetize the mice by injecting a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) in sterile saline by i.p. injection.

5. As soon as they are asleep (approx. 5 min. after anesthesia injection), thoroughly shave the backs of the mice (Oster animal clippers with #40 comb).

6. One at a time, place animals in appropriate burning template to give a 14-16% total body surface area scald injury according to their weight (see chart in procedure room), and proceed with the burn injury (or sham – dunking mice in room temp water). Make sure the shaved part of the back is “sealed” on the burning template by gently pressing the mouse down into the template.

7. Hold the template containing the animal in the water for 7 seconds. Quickly blot the mouse’s back on paper towels. Removing as much hot water as possible from the animal prevents further scalding. Put the mouse back in a clean cage.

8. As soon as possible after the injury, resuscitate each mouse with 1.0 ml of 0.9% sterile saline (i.p.), pre-warmed in 37C water bath, or on warming pads.

9. Keep cages on heating pads for the next 3-4 hours to help the mice recover from procedure. Mice can be returned to the mouse room once they are walking and fairly alert.

10. Place some food pellets on the bottom of each cage; the mice can’t stretch their necks as easily to reach for food after burn injury. Replace water bottles.
Kupffer Cell Isolation

Materials

100µm mesh filters
50mL conical tubes
gentleMACS Dissociator
gentleMACS C Tubes
Krebs-Ringer-Buffer (KRB): 154mM NaCl, 5.6mM KCl, 5.5mM Glucose, 20.1mM HEPES, 25mM NaHCO3, adjust to pH 7.4 with NaOH.
PEB buffer: 0.5% bovine serum albumin and 2mM EDTA in phosphate-buffered saline.
Dilute 1:20 in autoMACS Rinising Solution.
DNase I solution: 30,000 U/mL DNase I in KRB
Collagenase IV solution: 5,000 Collagenase Digestion Units (CDU)/mL in KRB
0.5M CaCl$_2$ solution
0.2M MgCl$_2$ solution
Red Blood Cell Lysis Solution

Centrifuge

Procedure

1. For each sample, mix 4.4mL KRB into a gentleMACS C tube and add 20µL CaCl$_2$ solution, 50µl MgCl$_2$ solution, 500µL Collagenase IV solution and 25µL DNase I solution. Incubate at 37°C for 30 minutes.
2. Remove whole liver and carefully separate the gallbladder. Mince liver tissue with razor blades.
3. Incubate minced tissue in a prewarmed collagenase IV solution for 30 minutes at 37°C.

4. Run C tube on gentleMACS Dissociator program “m_liver_01”.

5. Incubate samples for 30 minutes at 37°C under slow continuous rotation.

6. Run C tube on gentleMACS Dissociator program “m_liver_02”.

7. Pass contents of the C tube through a 100µm mesh filter that has been rinsed with PEB buffer. Collect in a 50uL conical tube. Rinse C tube with 5mL PEB buffer and pass through filter again. Wash the filter with 10 mL of PEB buffer collecting all rinses in the 50mL tube. Add 10mL more of PEB buffer.

8. Centrifuge suspension at 17xg for 4 minutes at 4°C to remove hepatocytes.

9. Collect supernatant and transfer to new 50mL tube that has been prerinsed with PEB buffer.

10. Centrifuge suspension at 300xg for 10 minutes at 4°C. Aspirate supernatant.

11. Resuspend cell pellet in 1mL PEB buffer and add 10mL Red Blood Cell Lysis Solution.

12. Incubate at room temperature for 5 minutes.

13. Add 30mL of PEB buffer and centrifuge at 300xg for 10 minutes at 4°C. Aspirate supernatant.

14. Resuspend cell pellet in 2mL of PEB buffer and adjust volume with PEB buffer to a final volume of 30mL.

15. Centrifuge at 300xg for 10 minutes at 4°C. Aspirate supernatant.

16. Resuspend in 90uL of PEB buffer per 10^7 cells and add 50uL of CD11b MicroBeads.
17. Mix well and incubate for 15 minutes at 4°C.

18. Wash cells by adding 10mL of PEB buffer and centrifuge at 300xg for 10 minutes at 4°C. Aspirate supernatant.


20. Prime autoMACS and exchange necessary reagents or magnetic columns.

21. Place tube containing magnetically labeled cells in the autoMACS separator and run on “Possel” for positive selection.

**Tail Vein Injection**

**Materials**

Hollow Styrofoam housing with a small hole in the side

27 gauge needle with syringe

Heating Pad

Ethanol (70% v/v)

**Procedure**

1. Place animal cage on warming pad.

2. Bend needle to a 120° angle with the syringe.

3. Place mouse under Styrofoam housing, pulling tail through the side hole.

4. Wet the tail with ethanol and rub proximal portion of the tail until a lateral tail vein is visible.

5. Insert needle into lateral vein and slowly inject drug or saline control.
ImageJ Analysis of Lung Tissue Histology

Materials

Lung H&E slides
Microscope with camera
ImageJ program

Procedure

1. Save 10 high power images per animal as jpeg files in a separate folder for each animal.
2. Import folder as an “Image Sequence” in the ImageJ program.
3. Convert to 8-bit and adjust contrast and threshold if necessary. Must apply the same settings to all sets of images.
4. Convert to binary.
5. Set measurements to be captured with threshold as area fraction.
6. Select “measure” and record.

Mesenteric Lymph Node Plating for Bacterial Translocation

Materials

1.5 mL tubes
RPMI with 5% FBS
Frosted glass slides
Small petri dishes
Tryptic soy agar plates
Cell spreaders
Procedure

1. Collect lymph nodes in a 1.5 mL tube with 0.5 mL RPMI (without antibiotic and with 5% FBS), add RPMI steriley to tubes on day of sac, keep on ice.

2. Separate lymph nodes from connective tissue and transfer to a new 1.5 mL tube containing 0.5 mL RPMI. (Lymph nodes should sink in RPMI.)

3. Transfer nodes (cut tip from a 1 mL pipette tip to allow transfer of nodes and RPMI) to the frosted side of one glass slide. Homogenize by crushing lymph nodes between the frosted sides of 2 glass slides over a small petri dish to collect the homogenate. Wash slides with 200 uL of RPMI and transfer collected homogenate from small petri dish to 1.5 mL epi tube.


5. Leave plates overnight in 37C incubator.

6. Count colonies at 24 hour.
REFERENCES


VITA

Michael M Chen attended the University of Wisconsin – Madison for his undergraduate where he obtained a Bachelor’s of Science in Medical Microbiology and Immunology. After graduation Michael spent four years at Kimberly-Clark Corporation as a scientist and product developer. In 2010 Michael matriculated into the Loyola University Chicago Stritch School of Medicine MD/PhD program. After completing two years of medical school, he began his graduate education in the Integrative Cell Biology Program under the mentorship of Dr. Elizabeth J Kovacs.

Michael’s dissertation work on alcohol’s modulation of the post burn hepatic response was supported by a predoctoral Ruth L. Kirchstein National Research Service Award from the National Institute of Health and a Research Seed Grant from the American Medical Association Foundation. Upon completion of his graduate studies, Michael will return to finish medical school.