Inebriated Immunity: Alcohol Affects Innate Immune Signaling in the Gut-Liver-Brain Axis

Patrick P. Lowe
University of Massachusetts Medical School

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INEBRIATED IMMUNITY: ALCOHOL AFFECTS INNATE IMMUNE SIGNALING IN THE GUT-LIVER-BRAIN AXIS

A Dissertation Presented

By

PATRICK PAUL LOWE

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

July 18, 2018

M.D., Ph.D. Program
INEBRIATED IMMUNITY: ALCOHOL AFFECTS INNATE IMMUNE SIGNALING IN THE GUT-LIVER-BRAIN AXIS

A Dissertation Presented
By

PATRICK PAUL LOWE

This work was undertaken in the Graduate School of Biomedical Sciences
M.D., Ph.D. Program
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July 18, 2018
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ABSTRACT

Alcohol is a commonly consumed beverage, a drug of abuse and an important molecule affecting nearly every organ-system in the body. This project seeks to investigate the interplay between alcohol’s effects on critical organ-systems making up gut-liver-brain axis.

Alcohol initially interacts with the gastrointestinal tract. Our research describes the alterations seen in intestinal microbiota following alcohol consumption in an acute-on-chronic model of alcoholic hepatitis and indicates that reducing intestinal bacteria using antibiotics protects from alcohol-induced intestinal cytokine expression, alcoholic liver disease and from inflammation in the brain. Alcohol-induced liver injury can occur due to direct hepatocyte metabolic dysregulation and from leakage of bacterial products from the intestine that initiates an immune response. Here, we will highlight the importance of this immune response, focusing on the role of infiltrating immune cells in human patients with alcoholic hepatitis and alcoholic cirrhosis. Using a small molecule inhibitor of CCR2/CCR5 chemokine receptor signaling in mice, we can protect the liver from damage and alcohol-induced inflammation. In the brain, we observe that chronic alcohol leads to the infiltration of macrophages in a region-specific manner. CCR2/CCR5 inhibition reduced macrophage infiltration, alcohol-induced inflammation and microglial changes. We also report that chronic alcohol shifts excitatory/inhibitory synapses in the hippocampus, possibly through complement-mediated remodeling. Finally, we show that anti-inflammasome inhibitors altered behavior by reducing alcohol consumption in female mice.
Together, these data advance our understanding of the gut-liver-brain axis in alcoholism and suggest novel avenues of therapeutic intervention to inhibit organ pathology associated with alcohol consumption and reduce drinking.
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<td>µg</td>
<td>Microgram</td>
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<td>µL</td>
<td>Microliter</td>
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<tr>
<td>Acox1</td>
<td>Acyl-coenzyme A oxidase 1</td>
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<td>Adipose differentiation-related protein</td>
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<td>Alexa fluor 647</td>
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<td>ALT</td>
<td>Alanine transferase</td>
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<td>APC</td>
<td>Allophycocyanin</td>
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<td>AST to platelet ratio index</td>
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<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing CARD</td>
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<td>AST</td>
<td>Aspartate transferase</td>
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<td>AUD</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>BEC</td>
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<td>CD</td>
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<td>CFU</td>
<td>Colony forming unit</td>
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<td>CXCL</td>
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<td>Death-associated protein</td>
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<td>ELISA</td>
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<td>Fetal bovine serum</td>
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<td>g</td>
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Green fluorescent protein</td>
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<td>Gastrointestinal</td>
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<td>Integrin subunit beta 2</td>
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<td>IU</td>
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<tr>
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<td>LDH</td>
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<td>Liver mononuclear cell</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LTCDS</td>
<td>Liver Tissue Cell Distribution System</td>
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<td>Ly6g</td>
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<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>Pair-fed</td>
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CHAPTER I

Introduction

Alcohol has saturated human society for millennia. Ancient Greeks and Romans worshiped gods such as Dionysus and Bacchus who were celebrated as patrons of the vine, of a good harvest and of winemaking. Alcohol, and wine in particular, are important features in many modern religions, including the Judeo-Christian tradition: in the Torah, Noah is credited with planting the first vineyard (Genesis 9:20) and in the Gospels, Jesus’ first public miracle was to turn water into wine during the Wedding at Cana (John 2:1-11). Indeed, alcohol permeates even our modern-day pop-culture, so much so that the British Medical Journal recently published a retrospective study of Commander James Bond (code name: 007) that assesses the likelihood for adverse medical outcomes associated with his high alcohol intake.

Despite the prominence of alcohol throughout ancient and modern human history, alcohol consumption has significant influence on health and disease. Upon introduction of alcohol into the body, its interaction with multiple organs can induce both anxiolytic and euphoric sensations as well as dangerously adverse effects. Beginning with the intestinal lumen and the gastrointestinal parenchyma, systemic interactions with the immune system, metabolism in the liver and then systemic circulation, critical contact points exist throughout the body where alcohol can influence our physiology. Given the near ubiquitous interactions alcohol has with the organs of the body, we propose that the
study of alcohol physiology and pathology requires a holistic approach, encompassing organs throughout the body. Previous research highlighting various mechanisms in the study of alcohol often emphasize contributions from the intestine, the liver, the immune system and the brain. Here, we will explore the hypothesis that immune activation and organ-specific pathology induced by alcohol consumption is dependent on cross-talk between the intestine, liver, peripheral immune system and the brain.

**Clinical manifestations of alcohol use disorder**

Alcohol use and abuse results in significant disease burden worldwide. In the United States, according to the National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health, 17 million adults and half a million adolescents have an alcohol use disorder². In 2016, the Centers for Disease Control and Prevention reported 42,000 Americans died due to opioid overdose³ while more than 80,000 patients die of alcohol related causes each year². Behind only tobacco and poor diet/physical activity, alcohol use disorder (AUD) is the third leading cause of preventable death² and a majority of patients with clinically diagnosed AUD do not receive treatment²,⁴,⁵. These data provide compelling evidence of the need for better understanding of alcohol use and related diseases, as well as the critical need for interventions to both reduce organ-specific disease and mitigate alcohol addiction.

Alcohol use disorder is described in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5) as problem drinking that leads to either clinically relevant or psychosocial functional impairment⁶. DSM-5 diagnosis of AUD is dependent on having
two or more of eleven symptoms. These symptoms include: being unable to cut back drinking despite a desire to do so, having memory blackouts due to excessive drinking, experiencing withdrawal symptoms when not drinking and drinking despite it causing troubles with family, friends or work. Severity of the disorder is defined by increasing symptom number6.

AUD has multiple pathologic medical sequelae in organs throughout the body. Common pathophysiologic manifestations of AUD, discussed in brief below, include disease of the gastrointestinal tract, alcoholic liver disease, cardiomyopathy, bleeding disorders, peripheral neuropathy, brain alterations and addiction7.

Alcoholic liver disease (ALD) is a prominent clinical concern because of the near ubiquitous nature of alcohol exposure in cultures across the globe8. ALD pathophysiology falls on a progressive spectrum of disease often related to the amount and duration of alcohol consumption. This spectrum includes liver steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma9. Some stages of disease are reversible, such as fatty liver, which can be corrected with abstinence. However, more severe forms of ALD develop in 35% of heavy alcohol drinkers and may be irreversible10. Hallmarks of ALD include hepatocyte injury (indicated by systemic release of liver enzymes such as alanine and aspartate transaminase (ALT/AST)), lipid accumulation, and immune cell infiltration.

Historically, AUD has been thought of as a psychiatric disorder that leads to organ disease. However, our scientific understanding of addiction has evolved and it is now recognized as a disease of the brain11. The underlying neurobiological mechanisms
of AUD included dysregulated neuronal activity and excitability, reward pathway activation and neurotoxicity in various brain regions. Neurologic and psychologic symptoms of AUD include intoxication (dyscoordination, dizziness, blackout, etc.), alcohol dependence and craving, memory deficits and withdrawal symptoms (including seizure, tremor, agitation, psychosis, etc.). Immune signaling has emerged as an important component underpinning many of these changes\textsuperscript{12-17} and will be explored further below.

AUD is associated with various other health problems and diseases beyond the liver and brain\textsuperscript{18}. In the gastrointestinal (GI) tract, gastritis, ulcers and pancreatitis are common. Cardiovascular diseases include cardiomyopathy and increased risk for bleeding. Peripheral neuropathy, sexual dysfunction, osteoporosis and increased risk of breast and GI cancers are just some of the remaining diseases associated with chronic alcohol abuse.

Current therapies to treat AUD vary in their therapeutic mechanism and include naltrexone (an opioid antagonist), acomporosate (a modulator of neurotransmitter signaling) and disulfuram (an inhibitor of acetaldehyde dehydrogenase)\textsuperscript{19}. However, millions of patients continue to suffer with AUD and methods to reduce alcohol consumption, whether through changes in behavior or pharmacologic therapies, can have a significant impact on relieving a preventable, but often untreated, health burden. For some of the sequelae of AUD, such as progressive alcoholic liver disease, there are no definitive therapies except abstinence, and current standards of care (with corticosteroids such as prednisolone) have limited efficacy, multiple contraindications and significant
side effects\textsuperscript{20}. These limited options make discovery of new treatments for AUD and the associated clinical diseases of great importance for both improved patient outcomes and reduced burden on healthcare systems and society at large.

Decades of research into AUD have pointed toward an axis of organs critical in the pathomechanisms of AUD-associated diseases. Based on the sequence of exposure to alcohol once it is consumed, these organs include the intestinal microbiome and the GI tract, the liver, the peripheral immune system and the central nervous system (CNS). The impact of alcohol on each of these organs in the development of disease is discussed below.

**Influence of alcohol on gut microbiome**

While the first organs that come to mind when thinking about AUD-associated disease may be the liver or the brain, the first organ that alcohol actually encounters after consumption is the GI tract. Consumed orally, alcohol is treated as much of all oral intake is treated: it is passed through the esophagus to the stomach and eventually to the intestines. Along the way, alcohol encounters the microbiome of the GI tract as well as the cells that make up the gut barrier. These interactions are critical first steps in our physiologic interaction with alcohol and varying degrees of homeostatic disruption contribute to the ensuing intoxication, dysfunction and ultimately, pathology\textsuperscript{21,22}.

For decades, researchers have observed an increase in endotoxin in the circulation of patients with alcoholic liver disease, a phenomenon referred to as alcohol-induced “leaky gut”\textsuperscript{23-27}. Endotoxin is a component of the gram-negative bacterial cell wall and is
also known as lipopolysaccharide (LPS). However, it was not immediately apparent how bacterial products (also referred to as pathogen-associated molecular patterns or PAMPs), such as LPS, could end up increased in the circulation. In 2000, Parlesak et al. in Germany reported that patients with alcoholic liver disease have increased permeability in the intestine to large molecular weight molecules of polyethylene glycol compared to control human subjects. Additionally, they reported 5-fold increases in plasma endotoxin in patients. This important demonstration of the loss of intestinal barrier integrity corroborates the phenomenon observed by others in both human disease and in animal models of AUD. Our lab recently showed that leaky gut is not restricted to patients with chronic alcohol use disorder, but can also be observed with a single alcohol binge in otherwise healthy control subjects.

The exact mechanisms involved in the breakdown of the intestinal barrier remain unclear, although multiple possible pathways have been proposed. Bacteria are capable of metabolizing alcohol and the alcohol metabolite acetaldehyde as well as production of reactive oxygen species cause disassociation of intestinal tight junction proteins. Multiple cytokines, including IL-1β, TNFα and IL-6, have been shown to reduce key proteins for tight junction integrity through activation of various intracellular signaling pathways and these cytokines, coming from peripheral immune cell activation, liver immune cells or from intestinal cells, are increased in the circulation of patients with AUD. NFκB, an important proinflammatory transcription factor, induces rearrangement of cellular cytoskeletal proteins. Trophic signaling as well as luminal microenvironment homeostasis involving mucins and lectins are also important in the
intestinal pathogenesis of alcohol use and changes in intestinal metabolites such as phenols and indole compounds have been reported after alcohol exposure. Given these various lines of evidence, it is likely that alcohol acts through a variety of mechanisms to induce cellular stress and barrier dysfunction that culminates in the leakage of bacteria and microbiome products from the lumen of the intestine through the intestinal lamina propria and into the circulation.

In addition to gut barrier breakdown, alcohol consumption induces dysbiosis of the gut microbiome and bacterial overgrowth in humans and rodents. Dysbiosis refers to alterations from the normal intestinal microbiome composition and is likely caused by some direct effect of alcohol, such as changes in gastric acid secretion leading to a shift in pH, or by the introduction of a new energy source, ethanol, which can be metabolized by some bacteria. A decrease in the rate of intestinal flow in AUD patients could also contribute to a microenvironment that favors some bacterial species over others. Dysbiosis is not limited to bacterial species content, however, as intestinal metabolites and metabolism of bile acids are also affected by chronic alcohol consumption. Some have suggested that it is the microbiome dysbiosis, or perhaps the associated shifts in gut lumen content (bile acids, metabolites, etc.), that primarily drives gut barrier leakage because enteric microbes can provide positive trophic signals to the barrier. However, studies have shown that after three weeks of alcohol detoxification, intestinal permeability is normalized despite the persistence of shifts in the microbiome content, suggesting that if the microbiome content does play a role in leaky gut formation, it is likely secondary to other causes.
The composition of the gut microbiome has a significant causative role in some diseases, such as inflammatory bowel disease\cite{56}, and dysbiosis is particularly prominent in ulcerative disease associated with *Helicobacter pylori* and *Clostridium difficile-* associated colitis. Similar to the influence of stomach acid on *Helicobacter pylori* infection, the microbiome composition may change secondary to environmental factors, such as the diet that intestinal bacteria are exposed to. One particular study in AUD patients highlights the important effects of diet, and in particular drink choice, on the microbiome composition\cite{30}. Researchers studying the intestinal bacterial content of AUD patients noted that only a subset of patients had altered bacterial composition and that this group of patients tended to consume more liquor compared to wine or beer.

To what degree the microbiome affects other organs leading to AUD pathology is an active area of research with recent findings that suggest that the microbiome has significant influence on the peripheral immune system, liver and the brain.

**The effect of the microbiome on peripheral immune cells, the liver and the brain**

The presence of a microbiome contributes critically to host immune development and defense. Germ-free mice have impaired peripheral and organ-specific immune development at the cellular and molecular levels\cite{57,58}. Importantly, it has been suggested that antibiotic treatment in human patients early in life, which can cause changes in the intestinal microbiome, may contribute to autoimmune and inflammatory diseases such as allergic asthma, multiple sclerosis and inflammatory bowel disease\cite{59-61}. 
Leakage of bacteria or their cellular components and products can occur in cases of intestinal barrier integrity loss and breakdown of natural lamina propria defense mechanisms. When this occurs, the leaked components enter circulation and can encounter immune cells in circulation or in organs throughout the body. This leakage has been implicated in numerous peripheral diseases, such as metabolic syndrome and diabetes, as well as psychiatric diseases including autism spectrum disorder, anxiety and depression.

The liver is the first organ to be encountered by blood flowing through the portal circulation from the intestine and therefore serves to remove or neutralize any bacteria, bacterial products and absorbed toxins that may have translocated across the intestinal barrier. Interestingly, bacterial contents within the intestine have a significant impact on alcohol-related liver function. The degree of cirrhosis in human AUD patients has been correlated with increasing bacterial overgrowth. Additionally, fecal transplantation from human patients with AUD to previously healthy germ-free mice resulted in the development of alcoholic liver disease.

Intestinal-derived bacterial products, not necessarily dependent on the makeup of intestinal bacteria, also play a critical role in the liver pathology associated with alcohol consumption and compound the alcohol-induced cell stress, hepatocyte death and release of multiple sterile danger signals that occurs independently from the influence of the microbiome. Bacterial LPS, leaking from inside the intestinal lumen into the portal circulation and traveling to the liver, signals through pattern recognition receptors such as Toll-like receptor 4 (TLR4). TLR4 activation leads to intracellular signaling and an
inflammatory response that can amplify ongoing hepatocyte stress and damage caused by alcohol metabolism.

We and others have shown the importance of intestinal-derived bacterial signaling for the induction of liver inflammation. Antibiotic treatment in certain mouse models of AUD reduces the bacterial load in the GI tract as well as circulating PAMPs such as LPS, thereby attenuating liver inflammation and steatosis after alcohol use. Treatment with antibiotics, dietary fiber, and probiotics, each of which impact gut microbiome composition, can protect from some of the hallmark features of alcoholic liver disease in animal models.

While previous studies have described the role of gut-derived bacteria in the liver, and others have studied how alcohol impacts the brain directly, the literature on what role the gut microbiota has regarding alcohol-induced CNS neuroinflammation is lacking. However, there is evidence from other disorders to suggest that the microbiome has effects on both addiction and neuroinflammation. Antibiotic treatment of mice, which dramatically reduced the total gut microbiome load, abrogated conditioned place preference for animals provided cocaine, suggesting a link between the gut microbiome and addiction. Additionally, recent evidence suggests that metabolites from the intestine can significantly affect microglia and alter neuroinflammatory signaling in mouse models of multiple sclerosis. Based on evidence from the gut-liver axis in AUD as well as from other disease models, we hypothesize that the intestinal microbiome may contribute to alcohol-induced neuroinflammation.
Alcohol’s effect on peripheral macrophages

Gut barrier breakdown and leakage of bacteria and PAMPs from the intestinal lumen is an important feature of alcohol-induced pathology\textsuperscript{24,26,27}. Circulating peripheral blood mononuclear cells (PBMCs), which include lymphocytes as well as monocyte/macrophages, respond to PAMPs by becoming activated and secreting cytokines. PBMCs taken from patients with alcoholism have an altered response to TLR ligands, exhibiting a blunted NFκB response, but an increased NLRP3 inflammasome response and increased production of IL-1β\textsuperscript{80}. In fact, patients in the first day or two of alcohol detoxification were shown to have elevated circulating IL-1β and IL-8, proinflammatory cytokines that positively correlated with symptoms of depression, anxiety and alcohol craving during the detox period. In the same patients, the anti-inflammatory cytokine IL-10 negatively correlated with these clinical scores\textsuperscript{80}. Patients with AUD also have elevated serum levels of TNFα and IL-6 that persists even after weeks of abstinence and these cytokines correlate with craving, depression and anxiety\textsuperscript{81}.

The response of alcohol-naïve human PBMCs to alcohol exposure reveals important insight into how immune signaling is affected by the duration of alcohol exposure. For example, acute alcohol decreases signaling through NFκB in human blood mononuclear cells as well as production of proinflammatory cytokines, such as TNFα and IL-6\textsuperscript{82,84}. However, chronic alcohol treatment of PBMCs increases signaling and cytokine production\textsuperscript{84,85}. Interestingly, while PBMCs from AUD patients have increased NLPR3 inflammasome response\textsuperscript{80}, PBMCs from healthy donors exhibit a blunted inflammasome response when exposed to alcohol\textsuperscript{86,87}. Upon alcohol consumption,
circulating PBMCs are therefore exposed to concentrations of alcohol that can directly influence their immune signaling in addition to any immune-activating gut-derived PAMPs or proinflammatory cytokines released from organs, such as the liver. Indeed, our lab has previously shown that exposure to acute alcohol and TLR ligands, such as LPS, can also increase proinflammatory cytokine production\textsuperscript{88}.

Alcohol itself and other microenvironmental factors significantly alter the immune response of circulating monocytes, which may influence their behavior once recruited to tissues to respond to a pathologic insult. This recruitment process typically occurs via release of chemokines, such as C-C chemokine ligand types 2 (CCL2; also known as monocyte chemoattractant protein-1 [MCP-1]) and CCL5 (also known as regulated on activation, normal T cell expressed and secreted [RANTES]), from tissues that attracts circulating monocytes via chemokine receptors, such as CC receptor type 2 (CCR2)\textsuperscript{89-91}. Within the liver, alcohol activates inflammatory signaling that includes upregulation of various cytokines and chemokines, including CCL2 and CCL5, leading to monocyte recruitment\textsuperscript{92}. Infiltration of peripheral monocytes has been shown to be a critical component of disease development as targeting CCL2/5 signaling (genetically or pharmacologically) in mouse models of ALD has shown protection from disease\textsuperscript{93-95}.

**Alcoholic liver disease**

Alcoholic liver disease (ALD) is a chronic progressive disorder associated with prolonged alcohol consumption in some patients with AUD. The progression of disease includes liver steatosis, steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma\textsuperscript{9}. 

Some of its features are irreversible at the more severe ends of the spectrum while early stage pathology, such as lipid accumulation, are reversible with even short-term abstinence\textsuperscript{10}. However, a portion of patients with AUD develop alcoholic hepatitis which can be rapid in onset and is associated with significant mortality and persistent inflammatory lesions\textsuperscript{10,96}.

Primary damage in the liver is due to alcohol and alcohol metabolism itself, which induces mitochondrial and endoplasmic reticulum stress and the release of reactive species damaging to hepatocytes, the main parenchymal liver cell\textsuperscript{70,71,97-99}. Alcohol-induced reactive oxygen species and shifts in NAD+/NADH balance can be toxic and lead to hepatocyte cell death\textsuperscript{100-102}. Hepatocytes can also be stimulated by gut-derived LPS to produce and release acute phase reactants such as C-reactive protein (CRP), ceruloplasmin, serum amyloid A and even proinflammatory cytokines\textsuperscript{103,104}.

A secondary insult that contributes significantly to ALD involves immune signaling. Immune activation is likely due to multiple factors, including hepatocyte stress and damage, changes in the gut microbiome and leakage of bacterial products, systemic cytokines and peripheral blood immune cell activation and other yet unknown signals\textsuperscript{105-107}. Many studies have described the variety of proinflammatory cytokines induced by alcohol in the liver as well as chemokines that attract monocytes, neutrophils and lymphocytes\textsuperscript{108}. Prominent among these chemokine molecules are CCL2, which attracts monocytes, and IL-8, a neutrophil chemoattractant, among others. Neutrophils are a classic component of acute alcoholic hepatitis observed in liver histology\textsuperscript{96}. Although the role of neutrophils is not fully determined, neutrophils in the liver negatively correlate
with clinical outcomes in patients with alcoholic hepatitis\textsuperscript{109} and animal models that reduce neutrophils have shown some protection from alcoholic liver disease\textsuperscript{110}.

Kupffer cells are the resident macrophages of the liver and serve a critical role in ALD. Both Kupffer cells and macrophages that infiltrate from circulation express TLR4, the major receptor for bacterial LPS. After leaking through the gut barrier and entering the portal circulation, LPS stimulates TLR4 on hepatic macrophages leading to NFκB activation and the production of proinflammatory cytokines\textsuperscript{70,71}. Kupffer cells also contribute to alcohol-induced liver steatosis through signaling dependent on CCL2 expression\textsuperscript{93} and CCL2 inhibition has been shown to protect from macrophage infiltration, proinflammatory cytokine expression and steatosis in models of chronic liver injury\textsuperscript{94}.

As resident immune cells, Kupffer cells express proteins involved in inflammasome signaling. The inflammasome is a protein assembly that senses molecular patterns through a receptor (for example, via the Nod-like receptor NLRP3), an adaptor (such as Apoptosis-associated speck-like protein containing a CARD, ASC) and an effector (such as the enzyme caspase-1). Inflammasome activation eventually leads to the cleavage and activation of IL-1β which may be released to spread a proinflammatory signal. Multiple studies have examined the role of inflammasome-related proteins and have shown that deficiencies in ASC, caspase-1 and IL-1 receptor ameliorate hallmarks of alcoholic liver disease in rodents\textsuperscript{70,111-113}. Treatment with a recombinant IL-1 receptor antagonist, thus blocking IL-1β signaling, also protects from key features of ALD\textsuperscript{112}. 
Liver fibrosis is a hallmark of end-stage ALD\textsuperscript{114}. In addition to Kupffer cells and other macrophages, the liver contains stellate cells which are responsible for the establishment of fibrosis by laying down scar tissue and increasing myofibroblast activity. Stellate cells become activated by LPS signaling, reactive oxygen species, cytokines from the circulation and Kupffer cells (particularly TGF$\beta$) and other mechanisms\textsuperscript{115}. Some studies suggest that the microbiome has influence over the progression of liver fibrosis and cirrhosis because bacterial overgrowth correlates with cirrhosis severity in AUD patients\textsuperscript{68,116}.

Through a combination of hepatocyte damage, immune cell activation and fibrosis, alcohol induces significant pathology in the liver that can lead to the development of steatosis, fibrosis and ultimately cirrhosis or hepatocellular carcinoma. Many pathways are involved in each of these pathophysiological processes, providing researchers many targets for the development of ALD therapeutics.

\textbf{Alcohol’s effect in the CNS}

For some, alcohol is a casual component of social and culinary life. However, for others, it is a drug of abuse and a substance of addiction. As proposed by Ron and Barak\textsuperscript{117}, the neural circuitry involved in alcohol addiction can be generally divided into two categories: go pathways and stop pathways. Go pathways promote alcohol consumption based on activation in the mesocorticolimbic system reinforcing learning, memory and reward\textsuperscript{118-120}. Stop pathways limit alcohol consumption and can be thought of as the negative aspects of alcohol intake. Stop pathway components contribute to the
aversive neurobiology of withdrawal and the physiological symptoms of a hangover. In some patients, an aversion to stop pathways and the rewarding benefits of go pathways lead to the development of alcohol use disorder\textsuperscript{117,121}.

The convergent roles of these seemingly divergent go/stop pathways in AUD are best exemplified in two pharmacological treatments. Targeting the go pathway, the opioid antagonist naltrexone reduces dopamine release and thus reduces the reward associated with alcohol consumption\textsuperscript{122}. Alcohol has been shown to activate components of the mesolimbic dopamine circuit which can be activated by $\mu$-opioid receptor activation that removes GABAergic inhibitory interneuron tone and allows ventral tegmental area (VTA) dopamine signaling\textsuperscript{119,120,123}. A recent study revealed that activation of dopamine signaling in regions such as the VTA influences the amount of alcohol consumed\textsuperscript{124}. VTA activation resulted in less alcohol consumption while high-consuming mice displayed VTA activation comparable to alcohol-naïve mice, suggesting underactive reward response may be, at least in part, responsible for alcohol seeking behavior. Some patients may be predisposed to benefit from opioid inhibition using the drug naltrexone, while others may be resistant to treatment. Genetic analysis of treatment susceptible/resistant patients reveals that mutations in the $\mu$-opioid receptor determine patient response to naltrexone\textsuperscript{125,126}. These clinical observations have been confirmed in nonhuman primates with comparable polymorphisms\textsuperscript{127}, highlighting the important variation of $\mu$-opioid receptor signaling and its effects on the mesolimbic dopamine reward circuitry in alcohol addiction within the population\textsuperscript{128}. 
Approved AUD therapies also target the stop pathway. Acomprosate is a partial agonist of the glutamate NMDA receptor that can serve to reduce receptor excitability and glutamate hyperactivity that occurs after alcohol cessation and is associated with withdrawal dysphoria, including in regions of mesolimbic reward circuit such as the nucleus accumbens\textsuperscript{129,130}. Disulfiram therapy may serve to augment the stop pathway, so much so that disulfiram-induced dysphoria associated with alcohol consumption (i.e. severe symptoms of a hangover) is better dealt with by alcohol cessation rather than repeated cycles of over intoxication and hangover\textsuperscript{131}. Disulfiram works by inhibiting complete alcohol metabolism, leading to a buildup of acetaldehyde and inducing the physiological symptoms of a hangover.

The effects of alcohol on the hippocampus and in particular on learning and memory have been studied for decades. A recent publication spanning 30 years showed that alcohol consumption led to increased odds of hippocampal atrophy in a dose-dependent manner\textsuperscript{13} and recent meta-analysis comparing alcohol consumption and hippocampal volume reveals a similar finding\textsuperscript{132}. The hippocampus is critical in the formation of memories and these findings of alcohol-associated decreased hippocampal volume manifest in memory dysfunction among alcohol consumers. Korsakoff’s syndrome is a well-described amnesic disorder in patients with AUD who have profound confabulatory speech and behavior associated with severe anterograde and retrograde memory dysfunction\textsuperscript{133}. Memory loss can also occur acutely and the age of onset of drinking correlates with an increase in acute-memory loss incidents (“blackouts”) even with reduced frequency in alcohol binge episodes\textsuperscript{134}. 
How alcohol can influence the hippocampus and memory formation is also an area of active research. Multiple studies have demonstrated that alcohol suppresses long-term potentiation (LTP) of synapses\textsuperscript{135-137}, a process associated with synaptic learning and memory formation, and some have suggested that this LTP inhibition is dependent on age with younger age associated with more inhibition\textsuperscript{137}. Alcohol consumption also significantly affects dendritic processes and synapses. For example, recent studies using mice that were pretreated with alcohol in adolescence revealed an increase in dendritic spines and a decrease in synapses in adulthood, suggesting dysregulation of dendrites and synapses within the hippocampus following alcohol exposure\textsuperscript{138,139}.

In addition to alterations in neurotransmission and reward pathways, alcohol has profound effects on immune signaling in the brain. Neuroimmune activation has been noted in post-mortem CNS tissue of patients with AUD\textsuperscript{140-143}. Various mechanisms are involved and likely act in concert to induce neuroimmune signaling in the setting of alcohol consumption. Alcohol induces oxidative stress by disrupting mitochondrial function in the brain which leads to the release of reactive species\textsuperscript{144}. Alcohol, along with neuroimmune activation, has also been demonstrated to induce neurodegeneration and reduce neurogenesis\textsuperscript{145-147}. Alcohol upregulates CNS expression of important neuroimmune receptors including TLR2, TLR4 and RAGE\textsuperscript{141,148} making the CNS primed for pattern recognition and immune activation. Alcohol-induced neuroinflammation and its role in pathology and behavior are emerging areas of intense study and represent important avenues for the development of novel therapeutics to assist in addiction therapy.
Microglia, neuroinflammation and peripheral macrophages

Microglia are derived from the hematopoietic lineage from the yolk sac and enter the brain during development around embryonic day 9.5-10.5 in mice. As the resident macrophages of the CNS, microglia serve multiple physiological functions including CNS surveillance, neuroimmune protection and synaptic maintenance. However, in a disease state or following a CNS insult, microglia can become activated, much like other tissue resident macrophages, with characteristic morphologic changes and the production and release of inflammatory signals such as cytokines. During homeostatic development as well as during a neuroinflammatory response, microglia express multiple phagocytic receptors. Microglia also express pathogen and danger signal receptors, such as TLRs that are critical in alcohol-induced neuroinflammation as well as NOD-like receptors such as NLRP3.

TLR4 is the major receptor recognizing bacteria-derived LPS as well as various endogenous danger signals including HMGB1. Mice deficient in TLR4 exhibit less microglia activation and lower expression of proinflammatory cytokines when treated with alcohol than TLR4 proficient animals. LPS does not significantly cross the blood-brain barrier under healthy conditions, however alcohol can cause a breakdown in blood-brain barrier integrity. Regardless of whether LPS crosses into the brain following alcohol exposure, data from TLR4 knockout mice certainly highlight the
importance of signaling through this LPS-recognition receptor in the induction of neuroinflammation after alcohol exposure.

In addition to signaling through TLR4, alcohol induces a substantial inflammatory response through the NLRP3 inflammasome. Inflammasome activation requires multiple signaling steps beginning with activation of intracellular NLRP3 by danger associated molecular patterns (DAMPs) such as uric acid, ATP and reactive oxygen species\textsuperscript{175,176}. NLRP3 then forms a multiprotein complex with the adaptor molecule ASC which leads to the cleavage of pro-caspase-1 to active caspase-1. Active caspase-1 is an enzyme capable of cleaving pro forms of the cytokines IL-1\textbeta and IL-18. Upon cleavage, IL-1\textbeta can be released from the cell and serve as a cytokine signal to surrounding tissue\textsuperscript{177,178}. IL-1\textbeta expression and release has been identified as a hallmark of alcohol-induced neuroinflammation\textsuperscript{170,179,180} and blockade of IL-1\textbeta signaling ameliorates alcohol-induced neuroinflammation\textsuperscript{162}.

Astrocytes are another important glial cell type in the CNS. Astrocytes serve multiple functions in the brain including neurotransmitter uptake, ion buffering, structural support and even serving immune functions. They produce and release inflammatory cytokines and become activated, similar to microglia, with altered morphology and characteristic protein expression changes\textsuperscript{181-183}. Postmortem human CNS tissue from patients with AUD reveal increased staining of astrocyte markers\textsuperscript{174}.

Various studies have looked at the effects of alcohol on cell models and have shown that alcohol exposure can induce an inflammatory response in rodent brain slices\textsuperscript{184-186} and in primary glial cells\textsuperscript{162,170,171,187,188}. Two recent publications explore the
transcriptional changes caused by in vivo alcohol in specific glial cells and reveal important and distinct roles for each cell type. In the study, mice were provided a two-bottle choice of alcohol every other day for sixty days. Microglia and astrocytes were isolated from the total brain and the cell-specific RNA was sequenced. Interestingly, while shifts in expression were noteworthy in both cell types, network analysis in the astrocyte transcriptome revealed an upregulation in genes related to extracellular matrix formation and maintenance as well as calcium signaling and homeostasis\textsuperscript{189}. Conversely, biological network analysis revealed microglial gene expression upregulation in multiple immune networks related to “innate immune signaling,” “toll-like receptor signaling,” “positive regulation of NFκB” and “regulation of TGFβ signaling”\textsuperscript{190}. Although this study uses only one model of AUD in mice, it reveals that between two glial cell-types, both of which can be involved in immune signaling, microglia predominate as the immune responders in the setting of chronic alcohol.

Microglia also serve as critical players in the maintenance and maturation of neuronal connectivity in the CNS. Throughout development, microglia participate in engulfing inactive and unnecessary synapses throughout the brain\textsuperscript{154,191}. This process of synaptic pruning uses the complement pathway, classically described in the context of inflammation and immune signaling. Complement proteins tag synapses for engulfment and microglia, expressing various complement receptors and other surface phagocytic proteins, respond by pruning unwanted synapse structures\textsuperscript{157,159}.

Disruption of homeostatic microglia behavior, for example by disturbing the microenvironment in the CNS, can have significant impact on neurodevelopment and
result in significantly dysfunctional behavioral outcomes\textsuperscript{191-193}. Very recently, acute alcohol binge administration in animals has been shown to activate microglia from their resting, homeostatic state and induce neuroinflammation. Interestingly, associated with this immune activation is altered synaptic development and pruning in the prefrontal cortex\textsuperscript{194,195}. Indeed, \textit{in vitro} assays exposing organotypic brain slices to a proinflammatory milieu with various cytokines affects both excitatory and inhibitory synapse density\textsuperscript{196} and alcohol induction of cytokine expression in the CNS is a well-documented phenomenon\textsuperscript{170,179,180,187,188}.

Another cell type important during neuroinflammatory processes are recruited peripheral macrophages. Systemic inflammation leads to microglial recruitment of peripheral macrophages to the CNS\textsuperscript{197} and subsequent upregulation of innate immune signals\textsuperscript{198}. The effects of peripheral macrophage infiltration on CNS inflammation are seen both in models of systemic infection (LPS injection) as well as systemic sterile inflammation (circulating cytokines such as TNF\textsubscript{α}, IL-1\textsubscript{β} and IL-6)\textsuperscript{199}. Infiltrating macrophages may be targeted to an area of tissue damage or respond to sights of ongoing neuroinflammation\textsuperscript{200}.

Macrophage infiltration normally occurs sparingly if at all in the healthy brain, but a recent study from researchers in Spain has revealed an important mechanism by which peripheral immune cells may be able to enter into the typically immune-protected CNS. Rubio-Araiz et al.\textsuperscript{174} showed reduced levels of key blood-brain barrier (BBB) proteins, including basal lamina protein, collagen-IV and claudin-5 in human post-mortem tissue from patients with AUD. They also describe an increase in the number of
CD45+ cells in the dorsolateral prefrontal cortex in these patients. To date, this report provides the most comprehensive evidence of BBB breakdown in human alcoholic patients. The authors also show that the pattern of decreased barrier integrity is also present in mouse models of alcohol consumption where they observed reduced hippocampal expression of laminin and collagen-IV along with increased IgG extravasation. Interestingly, these BBB changes, along with increased MMP9 activity and ERK1/2 activation, were abrogated in TLR4 knockout mice, suggesting an important component in this process is inflammation- and immune cell-related.

Reduced BBB integrity could allow for the infiltration of peripheral immune cells, such as monocytes, as well as the leakage into the CNS of proinflammatory molecules such as LPS or circulating cytokines. Mice lacking TLR4, which is expressed on both microglia and peripheral monocytes among other cells, are protected from the decreased expression of BBB junctional proteins and show less IgG extravasation from the circulation into the brain parenchyma\textsuperscript{174}, suggesting a causative role of TLR4-dependent inflammation in BBB breakdown\textsuperscript{201}. Additionally, cellular stress in brain endothelial cells, perhaps related to immune signaling or as a primary response to alcohol-induced oxidative stress, has been shown to alter tight junction proteins and allows for immune cell infiltration\textsuperscript{202-204}. Indeed, both alcohol and its metabolite acetaldehyde decreased collagen expression and increased expression of matrix metalloproteinases at endothelial cells associated with the neurovascular space\textsuperscript{204}.

**CCR2 axis and alcohol**
Breakdown in endovascular barriers may be an important step to allow for infiltration of circulating monocytes into tissue such as the brain, but a chemoattractant signaling process is also critical for attracting peripheral immune cells to the site of organ damage. Signaling through the chemokine receptors CCR2 and CCR5 has been shown to be important in the development of multiple liver diseases including non-alcoholic steatohepatitis, fibrosis and hepatocellular carcinoma. Previously, our laboratory has shown the critical role of CCL2 signaling in alcoholic liver disease. Mice deficient in CCL2 were protected from liver damage, steatosis and inflammation. Interestingly, CCR2-/- mice were not protected from liver injury and steatosis, suggesting that at least some components of alcoholic liver disease may be influenced by CCL2 signaling in a CCR2-independent manner.

CCR2/CCL2 axis signaling is also a common component of many CNS pathologies including hemorrhagic and ischemic stroke, epilepsy and ALS and rodents treated with CCL2 blocking antibody are protected from excitotoxic injury. Patients with AUD have significantly elevated levels of CCL2 protein in blood and cerebral spinal fluid, which persists for weeks following alcohol cessation and positively correlates with circulating liver enzymes. He and Crews have shown that CCL2 increases in limbic regions of the postmortem human brain, including VTA, SN, hippocampus and amygdala and rodent studies have described upregulation of CCL2 in various brain regions after alcohol administration. Indeed, intracerebroventricular administration of CCL2 in rats increased voluntary alcohol consumption and prenatal maternal alcohol consumption led to an increase in CCR2+
neurons in the lateral hypothalamus of offspring and also led to increased alcohol consumption in adolescence\textsuperscript{214} suggesting CCL2/CCR2 signaling may be related to addiction.

Researchers at the Scripps Institute have investigated the role of alcohol and CCL2 signaling. Using hippocampal slice cultures from transgenic mice that chronically overexpress astrocyte-produced CCL2, they show that elevated CCL2 did not affect baseline or alcohol-related synaptic transmission patterns. However, long-term potentiation, which is normally depressed under alcohol exposure, was restored in hippocampi with CCL2 overexpression\textsuperscript{215}. Interestingly, behavioral studies show that chronic CCL2 overexpression protects from alcohol-impaired cue and contextual fear conditioning and has effects on alcohol consumption, spatial learning and associative learning\textsuperscript{215,216}. These effects may be mediated by alterations in hippocampal synapses as CCL2 overexpression had modest effects on synapse protein expression in two-bottle choice alcohol feeding models\textsuperscript{217}.

CCL2/CCR2 signaling plays a critical role in regulating blood-brain barrier integrity and breakdown\textsuperscript{218}. In primary mouse endothelial cells, CCL2 treatment effectively reduced the integrity of an \textit{in vitro} BBB model by reorganizing tight junction proteins such as ZO-1, ZO-2, occludin and claudin-5 leading to reduced transendothelial electrical resistance and increased permeability. Barrier integrity was not affected in endothelial cells from CCR2\textsuperscript{-/-} mice\textsuperscript{219,220}. Additional research has shown that CCL2 cell-specific expression has important influence on the degree and time course of BBB breakdown during neuroinflammation\textsuperscript{221}. CCL2/CCR2 has been shown to be critical to
development of CNS neuroinflammatory disease in mouse models\textsuperscript{222} and in human patients\textsuperscript{223}.

While genetic manipulation of CCL2 and CCR2 is useful for studying mechanisms of peripheral monocyte recruitment to tissues, pharmacologic inhibition of this signaling axis provides a novel approach for mitigating disease progression. Cenicriviroc (CVC) is a small molecule inhibitor that blocks signaling through both the CCL2/CCR2 and CCL5/CCR5 signaling. These chemokine axes are involved in recruitment of monocytes and T cells\textsuperscript{224}. Accordingly, CVC has been shown to inhibit macrophage recruitment and activation in liver disease\textsuperscript{225}. A Phase 3 clinical trial (NCT03028740) exploring the efficacy and safety of CVC for the treatment of non-alcoholic steatohepatitis, a disease that involves significant inflammation\textsuperscript{178}, is currently underway. Testing CVC in a preclinical model of chronic alcohol consumption will aid in understanding the importance of peripheral monocyte recruitment and CCL2/CCR2 signaling in both alcoholic liver disease and neuroinflammation associated with chronic alcohol use.

Evidence of cytokines influencing behavior

Systemic inflammation outside of the CNS can be caused by viral or bacterial stimulation of the immune system or by sterile organ damage leading to the release of endogenous danger signals and immune activation. In either case, proinflammatory cytokines, chemokines and other inflammatory signaling molecules may be released into circulation and can affect the brain and behavior\textsuperscript{226}. Circulating cytokines can signal to
the CNS by crossing the BBB\textsuperscript{227,228}, signaling through neurovascular cells such as endothelial cells and pericytes\textsuperscript{229}, or even through the vagus nerve afferents that are disbursed throughout multiple peripheral organs, including the GI tract\textsuperscript{230,231}.

In the setting of alcohol consumption, gut barrier leakage of bacterial products coupled with hepatocyte metabolic stress in the liver lead to systemic release of inflammatory molecules, including cytokines, which can reach and influence the CNS\textsuperscript{232}. Cytokines in the CNS are broadly believed to induce a phenomenon known as sickness behavior which includes fever, behavioral changes (such as fatigue and social withdrawal) and an endocrine stress response\textsuperscript{233}. Indeed, inducing systemic inflammation can have significant behavioral outcomes as shown in studies in which human subjects were given an intravenous injection of LPS which induced circulating TNF$\alpha$, IL-6 and the neuroendocrine stress hormone cortisol. LPS-exposed subjects had more depressed mood, worse memory and increased anxiety\textsuperscript{234}. Although this study does not test the specific roles of each cytokine, it does highlight the important effect of LPS-induced immune activation. In patients suffering from depression associated with high circulating levels of proinflammatory cytokines\textsuperscript{233}, treatment with anti-inflammatory drugs (such as anti-TNF$\alpha$ therapy) improved symptoms\textsuperscript{235}.

Systemic cytokines have also been shown to correlate with psycho-behavioral symptoms associated with alcohol. Patients with alcoholism reported significant craving, depression and anxiety\textsuperscript{236} that correlated with circulating levels of proinflammatory cytokines\textsuperscript{80}. During alcohol detoxification, decreased craving symptoms correlated with decreasing expression of these same systemic inflammation markers\textsuperscript{29}. Glucocorticoid
therapy is often used clinically for its anti-inflammatory potential and a recent study in human patients with AUD showed that glucocorticoid treatment could reduce alcohol seeking behavior and consumption. Multiple anti-inflammatory agents, some of which are also antibiotics, influence alcohol consumption in mouse models. These include doxycycline and minocycline, which have been used to reduce alcohol-induced neuroinflammation and shown to reduce alcohol consumption in animal models. Naltrexone therapy, which has both opioid antagonistic as well as anti-inflammatory effects, also reduces alcohol consumption in human patients with AUD. The anti-inflammatory effects of naltrexone were demonstrated in studies using human peripheral blood cells and cell lines (including microglia) where naltrexone treatment inhibited IL-6 and TNFα production following stimulation of TLRs and inhibited microglial activation. Collectively, these studies reveal the important connection between the immune response and cytokine production caused by alcohol and the behavior symptoms seen in patients with AUD.

Addiction is now recognized as a disease of the brain with a cycle of intoxication, withdrawal and anticipation that has various neurobiological underpinnings, some of which are specific to the relevant drug of abuse. Based on gene expression studies of postmortem CNS tissue from patients with AUD, researchers identified immune pathways as neurobiological underpinnings of AUD and as potential targets for therapeutic intervention.

Animal studies can provide additional mechanistic evidence of the role of the immune system influencing alcohol-related behavior. Gene expression studies have
identified neuroimmune signaling as an important alteration in the CNS of mice after alcohol exposure\textsuperscript{244,245} as well as in alcohol-preferring mice compared to non-preferring mice\textsuperscript{246,247}. Interestingly, because some neuroimmune genes were found to be upregulated in mice selectively bred based on alcohol preference in previous generations, but not in response to any alcohol exposure in the tested mice themselves, these studies suggest that neuroimmune signaling may be involved in a predisposition to alcohol addiction rather than only a byproduct of chronic intoxication. Among these genes were members of the IL-1 family, components of NF\textsubscript{k}B signaling and IL-6 signaling pathways. Subsequent studies with animal models and knockouts for various immune-related genes, such as CD14 and IL-6, showed that indeed immune signaling influences alcohol-related behavior as knockout mice consumed less alcohol in a voluntary consumption assay\textsuperscript{16} whereas overexpression of cytokines, such as IL-6, increases alcohol preference\textsuperscript{248}.

Interestingly, related to observations from human patients with AUD that correlated alcohol-related symptoms with elevations in systemic inflammatory markers\textsuperscript{29,249}, researchers tested whether peripheral LPS exposure induced any changes in alcohol consumption in rodents. Peripheral LPS challenge has been shown in humans\textsuperscript{234,250} and in animal models\textsuperscript{251,252} to induce both systemic inflammation and CNS neuroinflammation. LPS-injected mice who were given free access to a choice of alcohol or alcohol-free drinking water consumed more alcohol than untreated mice\textsuperscript{253}. Unlike wild-type mice, mice deficient in CD14, which serves as a co-receptor molecule alongside TLR4 to recognize LPS, did not consume more alcohol than untreated CD14 KO mice, thereby emphasizing a connection between LPS-induced systemic
inflammation and alcohol drinking behavior\textsuperscript{254}. Interestingly, while TLR4 KO mice exhibit reduced sedation and motor function loss after alcohol exposure\textsuperscript{255}, multiple studies on the effect of TLR4 on alcohol consumption have revealed mixed results, suggesting that involvement of TLR4 immune signaling in alcohol seeking behavior may be more complicated than other immune signaling pathways\textsuperscript{174,254,255}.

To investigate a neural mechanism that could be manipulated by immune signaling and have an impact on behavioral response, researchers measured the firing rate of dopaminergic neurons in the VTA, a key reward center that has been implicated in alcohol and other drug addiction. Interestingly, they found that peripheral LPS treatment reduced dopaminergic neuronal firing\textsuperscript{253}. Activation of the VTA can also have a feed-forward effect and influence peripheral innate and adaptive immunity. Activation of dopaminergic neurons in the VTA was shown to enhance antibacterial activity in monocytes, macrophages and T cells following peripheral exposure to \textit{E. coli}\textsuperscript{256}. Chronic dysregulation of peripheral inflammation by alcohol as well as signaling in the VTA and elsewhere in the CNS due to the rewarding effects of alcohol likely induces critical adaptations over time. These studies provide insight into how systemic inflammation leads to changes in alcohol drinking behavior and how reward signaling connects with immune activation, emphasizing the importance of immune crosstalk between systemic organs, the peripheral immune system and the CNS.

\textbf{Conclusion}
Here, we have explored evidence that support the hypothesis that organ-specific immune activation and pathology induced by alcohol consumption is dependent on cross-talk between the intestine, liver, peripheral immune system and the central nervous system. The following chapters will provide additional data in support of this hypothesis and provide new insight into potential therapeutic targets.

The study in Chapter II describes the effect of alcohol on the gut microbiome composition in a new model of alcoholic hepatitis and neuroinflammation and how the use of antibiotics to reduce the gut bacterial load protects from inflammation in the small intestine, liver and brain and from features of alcoholic hepatitis in the liver.

Chapter III will explore how pharmacologic blockade of CCR2/5 signaling and inhibition of macrophage infiltration into the liver alleviates alcoholic liver disease in a mouse model of chronic alcohol.

Chapter IV presents new evidence of infiltrating peripheral macrophages into the CNS following chronic alcohol consumption in mice and the associated neuroinflammatory state induced by alcohol. Changes in excitatory and inhibitory synaptic densities are also described after alcohol consumption that are associated with increased expression of complement proteins and activated microglia.

Finally, Chapter V provides interesting insight into the behavioral effect of immune signaling on alcohol consumption from a study that reveals that inhibition of multiple steps in NLRP3 inflammasome activation reduces alcohol consumption in female, but not in male, mice.
Collectively, these data provide new insight into the complicated interactions between the gut, liver, peripheral immune system and the brain in the setting of alcohol and test multiple potential therapeutic approaches to protect from organ pathology and behavioral changes caused by alcohol consumption.
CHAPTER II

Alcohol-related changes in the intestinal microbiome contribute to small intestinal, hepatic and CNS inflammation

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Abstract

Alcohol-induced intestinal dysbiosis disrupts homeostatic gut-liver axis function and is essential in the development of alcoholic liver disease. However, the role of the intestinal microbiome in alcohol-induced neuroinflammation has yet to be defined. Here, we investigate changes in enteric microbiome composition in a model of early alcoholic steatohepatitis and dissect the pathogenic role of intestinal microbes in alcohol-induced intestinal inflammation, liver pathology and neuroinflammation. Wild type mice received a ten-day diet that was either 5% alcohol-containing or an isocaloric control diet plus a single binge. 16S rDNA sequencing defined the bacterial communities in the cecum of alcohol- and pair-fed animals. Some mice were treated with an antibiotic cocktail prior to and throughout alcohol feeding. Small intestine (SI) cytokines and inflammasome expression, liver neutrophils, cytokines and steatosis and neuroinflammation were evaluated. Acute-on-chronic alcohol administration induced shifts in various bacterial phyla in the cecum, including increased *Actinobacteria* and a reduction in *Verrucomicrobia* driven entirely by a reduction in the genus *Akkermansia*. Antibiotic treatment reduced the gut bacterial load and circulating bacterial wall component lipopolysaccharide (LPS) in alcohol- and pair-fed mice. Reduction in the gut bacterial load attenuated expression of alcohol-induced proinflammatory cytokines and increased inflammasome expression in the SI. Bacterial load suppression prevented alcohol-related increases in the number of myeloperoxidase- (MPO) positive infiltrating neutrophils in the liver. Expression of liver and circulating cytokines was also reduced in antibiotic-treated alcohol-fed mice. Alcohol-induced hepatic steatosis measured by Oil Red O
staining was significantly reduced in antibiotic-treated mice. Genes regulating lipid
production and storage were also altered by alcohol and antibiotic treatment.
Interestingly, antibiotic treatment did not protect from alcohol-induced increases in serum
aminotransferases (ALT/AST). Alcohol feeding significantly increased expression of
proinflammatory cytokines such as \textit{Tnfa}, \textit{Ccl2}, \textit{Hmgbl}, \textit{Il-17} and \textit{Il-23} in the brain and
antibiotic treatment attenuated expression. Alcohol feeding resulted in microglia
activation and morphologic changes in the cortex and hippocampus characterized by a
reactive phenotype. These alcohol-induced changes were abrogated following antibiotic-
induced reduction in the gut microbiome. Unexpectedly, antibiotic treatment increased
the mRNA expression of some inflammasome components in the brain. Our data indicate
that acute-on-chronic alcohol feeding alters the microflora at multiple taxonomic levels
and identifies loss of \textit{Akkermansia} as an early marker of alcohol-induced gut dysbiosis.
We conclude that gut microbes influence intestinal and liver inflammation, neutrophil
infiltration, liver steatosis and neuroinflammation following alcohol consumption and
these data further emphasize the role of the gut-liver-brain axis after alcohol
consumption.
Introduction

The National Institute on Alcohol Abuse and Alcoholism reports that 17 million adults in the US have an alcohol use disorder (AUD), approximately 1.4 million adults receive treatment for this disorder, and close to 88,000 people die each year from alcohol related causes\textsuperscript{257,258}. Chronic excessive alcohol use can lead to alcoholic hepatitis, a condition with high mortality, as well as neuroinflammation. The trigger(s) for acute alcoholic hepatitis and neuroinflammation are yet to be defined; however, the importance of the gut-liver-brain axis is increasingly recognized in AUD.

Animal and human studies have shown that alcohol consumption causes “leaky gut,” translocation of bacteria and microbial compounds such as lipopolysaccharide (LPS; also known as endotoxin), across the intestinal basement membrane into the portal and systemic circulations\textsuperscript{24,26,27}. Recently, we have shown that an acute dose of alcohol increases circulating markers of bacterial-product translocation across the gut barrier even in healthy individuals\textsuperscript{26}. Additionally, alcohol consumption leads to intestinal bacterial dysbiosis and bacterial overgrowth in the small intestine in humans as well as in mouse models of alcohol consumption\textsuperscript{30,46}. Once absorbed, alcohol along with gut-derived LPS are delivered via the portal circulation to the liver.

The liver is a primary site for alcohol metabolism and removal of bacteria and bacterial products that translocate across the intestinal barrier and into the portal circulation\textsuperscript{66,67}. Once in the liver, bacterial products (also known as pathogen associated molecular patterns or PAMPs) such as LPS activate Toll-like receptor 4 (TLR4) signaling pathways and initiate an innate immune response, thereby augmenting the damage caused
by alcohol’s primary insult on hepatocytes\textsuperscript{70,71}. The characteristic histological feature of inflammation in acute alcoholic hepatitis is the presence of neutrophils\textsuperscript{96}. While the exact role of neutrophils is yet to be defined in alcoholic liver disease (ALD), the clinical outcome in patients correlates with the presence of neutrophils in the liver in alcoholic hepatitis\textsuperscript{109}.

Various models replicate aspects of human ALD in mice. Among them, the acute-on-chronic feeding model utilizes a liquid diet containing 5% alcohol by volume for ten days followed by an acute binge\textsuperscript{259}. This model is emphasized for its replication of neutrophil infiltration in the liver, mimicking acute steatohepatitis in human patients\textsuperscript{110}. However, it is unknown if alcohol-related changes in the intestine, such as dysbiosis, are replicated in this new model.

LPS, unmetabolized alcohol and alcohol metabolites also travel beyond the liver and reach the systemic circulation and other organs, such as the central nervous system (CNS). While previous studies have investigated the direct effects of alcohol on the brain\textsuperscript{75-77}, little is known about the role of gut-derived microbial products and their impact on the nervous system and neuroinflammation.

Microglia play a critical role in sensing and responding to alcohol consumption and are involved in multiple immune signaling pathways\textsuperscript{142,161-163}. Microglia express TLR4, a pattern recognition receptor critical in alcohol-induced neuroinflammation\textsuperscript{14,165,166} as well as the NLR family pyrin domain containing 3 (NLRP3) inflammasome\textsuperscript{162}. Previous studies showed that TLR4 knockout mice are protected from increased cytokine expression in various regions of the brain and from
increased activation of microglia\textsuperscript{170-172}. TLR4 recognizes endogenous danger signals such as HMGB1\textsuperscript{168,169} and is the major pattern recognition receptor of bacterial LPS\textsuperscript{167}. Although LPS is not generally believed to cross the blood-brain barrier\textsuperscript{173}, data from TLR4 knockout mice suggests that signaling through TLR4 is an important component influencing alcohol-induced neuroinflammation. Neuroinflammation is mediated by the inflammasome complex, a multiprotein complex that senses pathogens and danger signals leading to cleavage and release of pro-inflammatory IL-1$\beta$ and IL-18\textsuperscript{162}.

Interestingly, antibiotic treatment in mice has previously been shown to reduce the bacterial load in the gastrointestinal tract (and thereby reducing LPS levels) coinciding with attenuation of liver inflammation and steatosis after alcohol use\textsuperscript{50,73}. This reduction in gut bacterial load could ameliorate the alcohol-induced changes in the brain. Antibiotic therapy is common for decompensated patients with alcoholic cirrhosis or hepatitis in order to treat infection in these immunocompromised patients. Minocycline and other derivatives, which act both as tetracycline antibiotics and as anti-inflammatory agents, have been used in preclinical studies and can reduce alcohol consumption\textsuperscript{239,260}. However, the use of broad-spectrum antibiotics to reduce the host microbiome and prevent the development of ALD has not been previously studied.

Therefore, we hypothesized that enteric bacterial products play an important role in acute alcoholic hepatitis and alcohol-induced neuroinflammation in the acute-on-chronic alcohol model in mice. Sequencing of enteric bacteria revealed modest shifts in composition after this short feeding model and identified changes in the genus \textit{Akkermansia} as an early indicator of alcohol-induced dysbiosis. We further postulated
that the gut-liver axis particularly affects the infiltration of neutrophils. We show that suppression of intestinal bacterial load with antibiotics reduced neutrophil infiltration and the inflammatory response in the liver. Following acute-on-chronic alcohol consumption in mice, we show that alcohol induces neuroinflammation in the CNS that is attenuated by antibiotic treatment. Interestingly, although cytokine expression was reduced, antibiotic treatment induced the mRNA expression of inflammasome components and cytokines processed by the inflammasome in the CNS. Together, these data offer critical information regarding the gut-liver-brain axis and its role in alcoholic liver disease in a newly described alcohol feeding model.
Methods

Mice and Alcohol Feeding

All animals were cared for in strict accordance with the approved Institutional Animal Care and Use Committee protocol specific to the procedures described in this study at the University of Massachusetts Medical School (Protocol #A-1154-14; G.S.). Wild-type C57BL/6J six- to eight-weeks-old female mice were purchased from Jackson Laboratories and were cohoused in the University of Massachusetts Medical School Animal Medicine Facility for one week prior to the start of the experiment at which time they were doubly housed. Mice were treated with an alcohol feeding model described by Bertola et al.259. Briefly, all mice were fed the Lieber-DeCarli pair-fed diet for five days to become acclimated to a liquid diet. Some mice were then switched to the Lieber-DeCarli alcohol diet containing 5% ethanol and maltose dextrin (to control for caloric intake). Pair-fed mice were calorie-matched with the ethanol-fed mice. On the tenth day, mice were gavaged between 12:00 AM and 2:00 AM with either ethanol (5g/kg body weight (BW)) or isocaloric maltose dextrin. Mice were cheek bled, anesthetized under ketamine (100mg/kg BW) and xylazine (10mg/kg BW) and then euthanized by exsanguination and bilateral pneumothorax at 9h post-gavage (9:00-11:00 AM). Throughout the experiments, animals were monitored at least twice daily (in the morning and the evening) by our laboratory as well as regular wellness checks by University of Massachusetts Medical School veterinary technicians and all efforts were made to minimize suffering. Following oral gavage, animals were monitored for respiratory distress, evidence of an accidental airway gavage. Four animals out of 50 exhibited
respiratory distress following an oral gavage (of either antibiotics or ethanol) and were euthanized according to our protocol.

16S rDNA Sequencing

Cecal contents were collected from some animals and frozen at -80°C. DNA was extracted using Stool DNA Extraction Kit (Qiagen) according to the manufacturer’s instructions. To check DNA quality and 16S content prior to sequencing, universal primers were used for SYBR Green quantitative polymerase chain reaction (qPCR; BioRad) with the following extended cycling protocol: 95°C 10min; 95°C 15sec, 60°C 30sec, 72°C 30sec for 40 cycles. Sequencing was completed at the Cincinnati University Children’s Hospital Medical Center’s DNA Sequencing and Genotyping Facility Core (Cincinnati, OH) as described261. All antibiotic-treated samples failed to yield 16S rDNA sequence data; one sample each from the ethanol- and pair-fed groups was excluded based on insufficient sequence data.

UPARSE262 and UTAX (http://www.drive5.com/usearch/manual/cmd_utax.html) were used to generate operational taxonomic unit (OTU) tables from 16S rDNA read data and to make taxonomic assignments. QIIME package scripts were used for calculations of α- (PD_whole_tree, chao1, observed_otus and shannon) and β- (Bray-Curtis, Un-Weighted UniFrac, and Weighted UniFrac) diversity263.

16S rDNA Sequencing data can be found in the NCBI GenBank repository with Accession Numbers: KY571432-KY572675.
Antibiotic Treatment and Bacterial Colony Count

Some mice were treated twice daily orally with an antibiotic cocktail containing ampicillin (100mg/kg BW; Sigma), neomycin (100mg/kg BW; Gibco), metronidazole (100mg/kg BW; Sigma) and vancomycin (50mg/kg BW; Sigma) beginning at the initiation of liquid diet and continuing until the oral ethanol or maltose dextrin gavage on the final feeding day. Non-antibiotic-treated mice received an equivalent volume of water. Gavage needles were rinsed with acid-treated water and sterilized in a hot-bead sterilizer between each mouse treatment to minimize transfer of microbes between animals.

Feces were collected directly from the anus prior to euthanization and frozen at -20°C until plating. Upon thawing, stool was immediately weighed, dissociated in liquid thioglycolate media (Sigma) and diluted 1:1000 prior to plating on non-selective agar plates (EMD Millipore). Plates were incubated at 37°C for 48h. Colonies were quantified using OpenCFU²⁶⁴ and were normalized to the mass of stool.

Histology

Oil Red O tissue staining on OCT-embedded frozen liver sections was completed and quantification was performed using ImageJ to assess hepatic steatosis. Formalin-fixed paraffin-embedded liver sections were stained at the University of Massachusetts Medical School Morphology Core with anti-mouse myeloperoxidase (MPO) antibody (Abcam) and subsequently labeled with streptavidin-biotin immunoenzymatic antigen for
detection with 3,3’-diaminobenzidine (DAB) (UltraVision Mouse Tissue Detection System Anti-Mouse HRP/DAB; Lab Vision).

Following sacrifice, brain tissue was dissected and fixed in 10% formalin overnight before paraffin embedding. Immunohistochemical staining was completed at the University of Massachusetts Medical School Morphology Core using anti-ionized calcium binding adapter molecule (IBA1) antibody (Wako; 1:1000) and subsequently labeled with streptavidin-biotin immunoenzymatic antigen for detection with 3,3’-diaminobenzidine (DAB) (UltraVision Mouse Tissue Detection System Anti-Mouse HRP/DAB; Lab Vision). Images were acquired from the described CNS areas by light microscopy (cortex; CA1, CA3 and dentate gyrus of the hippocampus) at 40X magnification for process length and cell body size measurements of microglia using ImageJ. IBA1 positivity was measured using the Color Deconvolution plug-in in ImageJ. The investigator was blinded to the sample groups during staining, image acquisition and ImageJ analysis.

**Biochemical Assays**

Serum was isolated from whole blood and frozen at -80°C until use. Serum alanine (ALT) and aspartate (AST) aminotransferases were measured using a kinetic method. Serum LPS was quantified using Pierce LAL Chromogenic assay (ThermoFisher). Serum CCL2 level was determined by enzyme-linked immunosorbent assay (ELISA; BioLegend).
Real-Time qPCR Analysis

RNA was extracted from liver tissue using RNeasy (Qiagen) according to the manufacturer’s instructions, including on-column DNase digestion (Zymo Research). cDNA was reverse transcribed from 1µg of RNA and then diluted 1:5 in nuclease-free water. SYBR Green (BioRad) real-time qPCR was performed according to the manufacturer’s instructions. The primers used are listed in Table 2.1 and 18S was used as a housekeeping gene for 2^{-ΔΔCt} method of RNA expression analysis. For 16S comparison between antibiotic-treated and non-treated animals, stool bacterial DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer’s protocol. After running a qPCR reaction using 16S primers similar to described above, a ΔCt was calculated using the average Ct value of each sample duplicate and subtracting the average ΔCt of untreated pair-fed mice. The bacterial 16S PCR product was run on a 1% agarose gel to visualize the relative reduction in bacterial load.

Table 2.1 Real-time PCR primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’&gt;3’)</th>
<th>Reverse (5’&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>GTA ACC CGT TGA ACC CCA TT</td>
<td>CCA TCC AAT CGG TAG TAG CG</td>
</tr>
<tr>
<td>16S</td>
<td>TCCTACGCGGGAGGCAGCATG</td>
<td>GGACTACCAGGGGTATCTAATCCTGT TT</td>
</tr>
<tr>
<td>E-selectin</td>
<td>ATG CCT CGC GCT TTT TCT C</td>
<td>GTA GTC CCG CTG ACA GTA TGC</td>
</tr>
<tr>
<td>Ly6g</td>
<td>TGC GTT GCT CTG GAG ATA GA</td>
<td>CAG AGT AGT GGG GCA GAT GG</td>
</tr>
<tr>
<td>Mpo</td>
<td>CAT CCA ACC CTT CAT GTT CC</td>
<td>CTG GCG ATT CAG TTT GG</td>
</tr>
<tr>
<td>Tnfa</td>
<td>GAA GTT CCC AAA TGG CCT CC</td>
<td>GTG AGG GTC TGG GCC ATA GA</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>ACT GCA CCC AAA CCG AAG TC</td>
<td>TGG GGA CAC CTT TTA GCA TCT T</td>
</tr>
<tr>
<td>Ccl2</td>
<td>CAG GTC CCT GTC ATG TTT C</td>
<td>TCT GGA CCC ATT CTT TCT TG</td>
</tr>
<tr>
<td>Il-1β</td>
<td>TCTTTGAAGTTGACGGACCC</td>
<td>TGAGTGAATACTGCCTGCCTG</td>
</tr>
<tr>
<td>Il-17</td>
<td>CAGGGAGAGCCTTCATCTGTGT</td>
<td>GCTGACCTTGGAGGATGAT</td>
</tr>
<tr>
<td>Il-23</td>
<td>AAGTTTCTCTTCTCCCTTCCCTGTGC</td>
<td>TCTTTGTGGAGCACAGCATGAG</td>
</tr>
<tr>
<td>Hmgb1</td>
<td>CGCGGAGGAATCAACTAA</td>
<td>TCATAACGACCGCTTGTAGC</td>
</tr>
</tbody>
</table>
The above forward and reverse sequences of primers were used in real-time PCR. Ly6g, Lymphocyte antigen 6 complex locus G6D; Mpo, myeloperoxidase; Tnfα, tumor necrosis factor-α; Cxcl1, C-X-C motif chemokine ligand 1; Ccl2, C-C motif chemokine ligand 2; Il-1β, interleukin-1β; Il-17, interleukin-17; Il-23, interleukin-23; Hmgb1, high-mobility group box 1; Nlrp3, NLR family pyrin domain containing 3; Asc, apoptosis-associated speck-like protein; Casp1, caspase-1; Il-18, interleukin-18; Fasn, fatty acid synthase; Ucp1, uncoupling protein 1; Prdm16, PR-domain zinc finger protein 16; Scd2, stearoyl-CoA desaturase 2; Adrp, adipose differentiation-related protein; Lcn2, Lipocalin-2.

### Statistical Analysis

Statistical analysis was carried out using GraphPad Prism Version 7.0 using Mann-Whitney test. $p < 0.05$ was considered statistically significant. Outlier exclusion was calculated using Grubbs’ outlier test with alpha set to 0.05.
Results

Alpha- and beta-diversity of cecal microbiome are unaltered by acute-on-chronic alcohol feeding

We applied 16S rDNA sequencing to define and to assess the changes in the cecal microbiota induced by the acute-on-chronic alcohol feeding in mice (Fig 2.1A). The bacterial load was substantially reduced in antibiotic-treated mice and 16S rDNA sequence was not obtained for that cohort. Of the non-antibiotic-treated mice, samples from 7/8 pair-fed (PF) and 9/10 alcohol-fed (EtOH) mice were available for analysis. We observed no significant differences (p>0.05) in \( \alpha \)-diversity between pair-fed and alcohol-fed groups (Table 2.2), indicating that the mean species diversity was not affected by acute-on-chronic alcohol.

Table 2.2 \( \alpha \)-diversity of cecal bacterial content.

<table>
<thead>
<tr>
<th></th>
<th>PD_whole_tree</th>
<th>chao1</th>
<th>observed_otus</th>
<th>shannon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Error</td>
<td>Average</td>
<td>Error</td>
</tr>
<tr>
<td>Pair-fed</td>
<td>6.76</td>
<td>2.35</td>
<td>82.38</td>
<td>36.25</td>
</tr>
<tr>
<td>EtOH-fed</td>
<td>7.37</td>
<td>2.07</td>
<td>87.86</td>
<td>31.59</td>
</tr>
</tbody>
</table>

Single rarefaction at a depth of 10,000 sequences indicated no significant difference in \( \alpha \)-diversity between pair- and ethanol-fed cecal bacterial communities by four different metrics.

Next, we examined \( \beta \)-diversity to assess the degree of dissimilarity of bacterial communities between samples. These analyses determined no significant differences between pair-fed and alcohol-fed mice. Additionally, comparisons within pair-fed and within alcohol-fed conditions revealed that the \( \beta \)-diversity within each group (intra-group
diversity) was not statistically different from diversity between the groups (inter-group diversity).

Alcohol induces taxonomic shifts in bacterial communities

Although α- and β-diversity were not significantly changed, we hypothesized that there may be specific taxonomic shifts after acute-on-chronic alcohol administration between alcohol-fed and pair-fed mice and 16S sequencing indeed revealed multiple taxonomic changes (Fig 2.1B-C). Compared to pair-fed mice, the phylum *Actinobacteria* was significantly enriched (Fig 2.1D; d_Bacteria:p_Actinobacteria, PF: 0.29% vs EtOH: 1.43%), while the phylum *Tenericutes* was reduced in relative abundance in alcohol-fed mice (Fig 2.1D; d_Bacteria:p_Tenericutes, PF: 0.14% vs EtOH: 0.00%). The most abundant phylum, *Verrucomicrobia* (Fig 2.1D; d_Bacteria:p_Verrucomicrobia) showed the greatest reduction in alcohol-fed mice (PF: 39.54% vs EtOH: 20.64%).
Figure 2.1 Alcohol induces shifts in gut bacterial content at various taxonomic levels. A) Mice were provided a liquid diet for five days to acclimate to the new diet. Some mice were then provided 5% ethanol in the liquid diet or provided a calorie-matched pair-fed diet for ten days. Nine hours before sacrifice, mice were orally gavaged with either ethanol (5g/kg BW) or a calorie-matched gavage of maltose dextrin. B) Cecal stool content was obtained from pair-fed (PF) or alcohol-fed (EtOH) mice and DNA was isolated for 16S bacterial sequencing. Following alignment and clustering of sequences, a cladogram was generated to show the bacterial taxa that are enriched in PF (green) or EtOH (red) mice. C) Proportional contributions of various bacterial phyla to the overall gut microbiome in PF and EtOH mice. D) The average percent of total bacteria measured
for each phylum reveals enrichment in Actinobacteria (navy blue) in the microbiome of alcohol-fed mice. Phylum Tenericutes (beige) was reduced in relative abundance in alcohol-fed mice. The phylum Verrucomicrobia (light blue), which represented the majority of bacteria in PF mice, was reduced in the acute-on-chronic alcohol model. The prefix letter followed by an underscore represents the taxonomic level (e.g. “d_” for domain, “p_” for phylum, etc.). Data are mean +/- SEM, n=8-9 mice/group. *p < 0.05 by Mann-Whitney test.
An examination of the nine most abundant families represented in our sample set (Fig 2.2A) revealed that the most abundant family in cecal content was *Verrucomicrobiaceae*, which was significantly reduced in alcohol-fed animals when compared to the pair-fed group. Other families with reduced abundance in alcohol-fed mice included *Lachnospiraceae* and *Moraxellaceae* (green asterisk), while *Eubacteriaceae* was enriched in alcohol-fed mice (red asterisk). Examination of the genus composition of the *Verrucomicrobiaceae* family revealed only one genus, *Akkermansia* which accounted for the reduction observed in alcohol-fed mice from the phylum (*Verrucomicrobia*) to the genus level (Fig 2.2B). This analysis identifies reduction of *Akkermansia* as an early marker of alcohol-induced changes in the gut microbiome.
Figure 2.2 Alcohol alters family-level community representation in some of the nine most abundant bacterial families. A) To represent a subset of the bacterial composition in cecal content of pair- (PF) and alcohol-fed (EtOH) mice, we chose the nine most abundant families, each comprising >0.5% of all families. Those differentially enriched in pair-fed mice are indicated by a green asterisk; red asterisk denotes families enriched in alcohol-fed mice. B) The genus Akkermansia represented 100% of the family Verrucomicrobiacea, the most abundant family found in pair-fed mice and was significantly reduced by alcohol administration. The prefix letter followed by an underscore represents the taxonomic level (e.g. “p_” for phylum, “c_” for class, etc.). Data are mean +/- SEM, n=8-9 mice/group. *p < 0.05 by Mann-Whitney test.
Antibiotic treatment dramatically decontaminates gut bacterial load

While the modulating effects of chronic alcohol administration have been studied in the gut microbiome, alcoholic liver disease and neuroinflammation, it is unclear how shorter alcohol use and/or alcohol binge affect inflammation signaling and what role the gut microbiome plays in this process. In this study, mice received 5% alcohol (EtOH) in a liquid diet for ten days (after a five-day liquid diet acclimation period), followed by a one-time alcohol binge or a calorie-matched pair-fed (PF) diet. To directly test the impact of gut bacterial load on alcohol-induced inflammation, we first used an antibiotic cocktail (ampicillin, neomycin, vancomycin and metronidazole) to drastically reduce the intestinal bacterial flora. In a preliminary study, we found that mice became averse to drinking water containing the dissolved antibiotic cocktail, which has also been observed by others. We therefore switched to a twice-daily oral gavage regimen to ensure adequate bacterial clearing.

Oral antibiotic treatment (Abx) caused a significant reduction in LPS in the circulation at the time of sacrifice both in pair-fed and alcohol-fed mice. The expression of 16S bacterial DNA, measured from mice stools collected immediately prior to sacrifice, was dramatically reduced by antibiotic treatment. Stool bacteria cultured on non-selective agar plates also revealed almost complete elimination of culturable colonies after five days of antibiotic treatment. Some recovery of bacteria in the stool was observed by the conclusion of the fifteen-day study, likely due to the development of antibiotic resistance. However, bacterial colony forming units (CFUs) were dramatically reduced in the stool obtained on the day of sacrifice in
antibiotic-treated animals compared with untreated mice (Fig 2.3F). Importantly, antibiotic treatment did not affect daily alcohol diet intake (EtOH-fed: 9.1 ± 0.6 mL/day/mouse; EtOH-fed plus antibiotics: 8.7 ± 0.2 mL/day/mouse; p=0.14). Together, these data indicate that antibiotic treatment successfully suppressed gut bacterial load and reduced circulating endotoxin in both pair- and alcohol-fed mice.
Figure 2.3 Oral antibiotics significantly reduce the gut bacterial load. A) Four groups of wild-type C57BL/6J female mice were treated with: pair-fed diet (PF; n=5); 5% alcohol diet (EtOH; n=10); oral antibiotics (Abx) with PF (n=6); or Abx with EtOH (n=9). An acute sugar or alcohol binge was given 9h before sacrifice. B) Serum endotoxin was measured at sacrifice to determine translocation of gut bacterial products into systemic circulation. C) DNA was isolated from the stool of PF and EtOH mice before sacrifice and 16S DNA was measured by qPCR using universal 16S primers. D) The PCR products from C) were run on an agarose gel for a general comparison of the four groups. E) Stools were resuspended in thioglycolate and plated on non-selective agar to measure gut bacterial load prior to antibiotic treatment (Untreated), after 5 days of Abx treatment (Abx Day 5) and at the end of the experiment (Abx Day 15). F) Colony forming units (CFUs) were quantified from stool extracted at sacrifice on Day 15. Data are mean +/- SEM, n=5-10 mice/group. *p < 0.05; n.s., not significant by Mann-Whitney test.
Alcohol-induced cytokine expression in the small intestine is attenuated by antibiotic administration

Previous studies have shown that intestinal cytokine expression can reduce gut barrier integrity and may allow leakage of pathogen-associated molecules from the intestinal lumen into the systemic circulation. Therefore, we measured the intestinal expression of various proinflammatory cytokines and found that they were increased after acute-on-chronic alcohol administration compared to calorie-matched pair-fed mice (Fig 2.4). The expression of $\text{Tnfa}$, $\text{Ccl2}$ and $\text{Hmgb1}$ mRNA was significantly increased following alcohol consumption and $\text{Il-17}$ and $\text{Il-23}$ expression also showed an increasing trend in alcohol-fed mice. Treatment with the antibiotic cocktail reduced the bacterial load in the intestine (Fig 2.3) and led to significantly attenuated alcohol-induced $\text{Ccl2}$ and $\text{Hmgb1}$ mRNA levels. Antibiotic treatment reduced the baseline expression of the inflammatory cytokines including $\text{Tnfa}$, $\text{Il-17}$ and $\text{Il-23}$ in PF mice compared to untreated pair-fed mice (Fig 2.4). Interestingly, even with antibiotic treatment, alcohol feeding still increased expression of $\text{Tnfa}$, $\text{Il-17}$ and $\text{Il-23}$ in the small intestine of antibiotic-treated mice compared to antibiotic-treated pair-fed mice (Fig 2.4).
Figure 2.4 Alcohol-induced small intestinal inflammation is reduced with gut bacterial load reduction. Expression of proinflammatory cytokines \( Tnf\alpha \), \( Ccl2 \), \( Hmgb1 \), \( Il-17 \) and \( Il-23 \) was measured from the small intestine of pair-fed (PF) or alcohol-fed (EtOH) mice with or without daily antibiotic treatment (Abx). Data are mean +/- SEM, \( n=4-10 \) mice/group. *\( p < 0.05 \) by Mann-Whitney test.
Antibiotic treatment increases the mRNA expression of the inflammasome in the small intestine

Recent research has highlighted an important connection between the intestinal microbiome and inflammasomes, particularly the NLRP3 inflammasome. Therefore, we investigated whether antibiotic decontamination of the gut impacted expression of inflammasome components in the small intestine. Expression of \textit{Il-1\beta}, \textit{Il-18}, \textit{Asc} and \textit{Casp1} mRNA was elevated in antibiotic-treated, alcohol-fed mice compared to untreated pair-fed mice. Alcohol consumption increased \textit{Nlrp3}, \textit{Asc} and \textit{Casp1} expression in the small intestine. Although antibiotic treatment in alcohol-fed mice significantly inhibited \textit{Il-18} mRNA levels and increased \textit{Asc} expression compared to untreated alcohol-fed mice, the expression levels of \textit{Il-1\beta}, \textit{Casp1} and \textit{Nlrp3} were not significantly affected.
Figure 2.5 Antibiotic treatment increases expression of inflammasome components and inflammasome-processed cytokines in the small intestine. Expression of inflammasome components *Nlrp3*, *Asc* and *Casp1* as well as the cytokines *Il-1β* and *Il-18* were measured from the small intestine of pair-fed (PF) or alcohol-fed (EtOH) mice with or without daily antibiotic treatment (Abx). Data are mean +/- SEM, n=4-10 mice/group. *p < 0.05 by Mann-Whitney test.
Neutrophil infiltration to the liver is attenuated in mice with reduced gut bacterial load

Having established a reduction in bacterial load and circulating LPS with antibiotic treatment, we hypothesized that this might reduce hepatic inflammation. To test this, we measured liver mRNA expression of the neutrophil-associated genes including E-selectin (associated with neutrophil attraction), Ly6g (a neutrophil-specific marker) and Mpo (an enzyme involved in processing phagocytic material in neutrophils). E-selectin was not significantly increased by alcohol, although antibiotics reduced its expression and alcohol-fed antibiotic-treated mice did display an increase when compared to pair-fed plus antibiotics (Fig 2.6A). The trend toward increased Ly6g expression following alcohol administration (p=0.052) was not abrogated after gut decontamination by antibiotics in alcohol-fed mice (Fig 2.6B). Liver Mpo mRNA expression was reduced by antibiotics in both control and alcohol-fed mice (Fig 2.6C). The increased involvement of neutrophils in the liver following alcohol consumption was confirmed by anti-MPO immunohistochemical staining. Pair-fed mice treated with antibiotics had fewer MPO+ neutrophils at baseline (p=0.10) and antibiotic-treated mice were protected from alcohol-induced increase in infiltrating neutrophils in the liver (Fig 2.6D-E).
Figure 2.6 Alcohol-induced hepatic inflammation and neutrophil infiltration is attenuated in mice with reduced gut-bacterial load. A-C) Liver expression of neutrophil associated genes E-selectin, Ly6g and Mpo were measured by qPCR and normalized to 18S. E) MPO immunohistochemical staining to detect neutrophils in the liver parenchyma revealed an increased number of neutrophils/area in alcohol-fed mice compared to pair-feds and reduced gut bacterial load led to fewer infiltrating neutrophils. Interestingly, antibiotics also cause a decreased trend in hepatic neutrophils at baseline in pair-fed mice (quantified in D)). Data are mean +/- SEM, n=4-10 mice/group. *p < 0.05; n.s., not significant by Mann-Whitney test.
Gut decontamination reduces alcohol-induced inflammatory mediators in the liver and circulation

Liver inflammation is a major component of the pathomechanism of alcoholic hepatitis in humans and in animal models. To test if liver inflammation is mediated by bacterial load, we measured transcripts of key cytokines and chemokines from liver tissues by qPCR. Expression of \( \text{Tnfa, Cxcl1} \) and \( \text{Ccl2} \) mRNA was elevated in alcohol-fed mice compared to pair-fed controls (\( p<0.05 \) for \( \text{Tnfa, Ccl2} \); \( p<0.1 \) for \( \text{Cxcl1} \)). Importantly, increases in \( \text{Tnfa} \) and \( \text{Cxcl1} \) were eliminated by antibiotic treatment in alcohol-fed mice (Fig 2.7A-C). Circulating CCL2 protein in the serum showed an alcohol-induced increase that was mitigated by antibiotic reduction of intestinal bacteria suggesting that gut decontamination has an important effect on liver inflammation in alcoholic hepatitis (Fig 2.7D). Together, this data showed that treatment with antibiotics reduced circulation of CCL2 and expression of inflammatory markers.
Figure 2.7 Antibiotic treatment reduces expression of inflammatory mediators in the liver and circulation. A-B) Liver expression of the cytokine \textit{Tnf\alpha} and the chemokine \textit{Cxcl1} is increased by alcohol and antibiotics eliminate these increases. C) Although mRNA levels of \textit{Ccl2} are not different between the antibiotic-treated/untreated alcohol-fed groups, D) circulating CCL2 protein is reduced in alcohol-fed antibiotic-treated mice. Data are mean +/- SEM, \(n=4-10\) mice/group. *\(p<0.05\); n.s., not significant by Mann-Whitney test.
Suppressed bacterial load reduces alcohol-induced liver steatosis but not serum transaminases

Antibiotic treatment resulted in proinflammatory signal reduction, suggesting that perhaps other aspects of alcoholic liver disease, such as hepatic steatosis, may also be impacted. To test this, we used Oil Red O staining and observed that alcohol-fed mice had increased hepatic steatosis compared to pair-fed mice. However antibiotic treatment reduced the alcohol-related increase in steatosis (Fig 2.8A-B). To investigate the effect of alcohol and antibiotic treatment on lipid regulation and synthesis, we measured mRNA levels of various liver genes involved in lipid metabolism (Fig 2.8C). Alcohol feeding reduced expression of fatty acid synthase (Fasn), uncoupling protein-1 (Ucp1), PR-domain zinc finger protein 16 (Prdm16), stearoyl-CoA desaturase 2 (Scd2). Antibiotic treatment, irrespective of alcohol feeding, also reduced expression of Fasn, Ucp1, and Prdm16 while antibiotics plus alcohol further reduced Scd2 compared to antibiotics plus pair-fed. Adipose differentiation-related protein (Ardp) is a molecule that coats intracellular stores of lipid and lipocalin-2 (Lcn2) is involved in intracellular lipid transport. Ardp expression was elevated in alcohol-fed animals regardless of antibiotic treatment and Lcn2 trended toward an increase (p=0.10) in alcohol-fed animals and was significantly increased in alcohol plus antibiotic mice, reflecting the increase in liver Oil Red-O in alcohol-fed animals. Serum ALT (Fig 2.8D) and AST (Fig 2.8E) were elevated in mice fed alcohol compared to those receiving control diet, indicating hepatocyte injury. Interestingly, serum ALT and AST levels remained elevated in ten-days plus
binge alcohol-fed mice receiving antibiotic treatment, suggesting the presence of microbe-independent mechanisms of alcohol-induced hepatocyte injury.
Figure 2.8 Steatosis but not serum transaminase increase from alcohol consumption is reduced by gut sterilization. A-B) Oil Red O staining on liver sections reveals that alcohol causes a dramatic increase in hepatic steatosis. C) Liver mRNA expression of various genes involved in lipid metabolism, including fatty acid synthase (*Fasn*), uncoupling protein-1 (*Ucp1*), PR-domain zinc finger protein 16 (*Prdm16*), stearoyl-CoA desaturase 2 (*Scd2*), adipose differentiation-related protein (*Ardp*) and lipocalin-2 (*Lcn2*) were measured by qPCR. D-E) Serum alanine (ALT) and aspartate (AST) transaminases, which are increased by acute-on-chronic alcohol, are unchanged by antibiotic treatment. Data are mean +/- SEM, *n*=4-10 mice/group. *p* < 0.05. For C), groups that do not share a letter are significantly different by Mann-Whitney test.
Antibiotic treatment abrogates alcohol-induced proinflammatory cytokine expression in the cerebral cortex

Chronic alcohol use results in neuroinflammation both in humans and in mice\textsuperscript{142,165}. We found that the ten days of chronic alcohol feeding followed by a one-time binge in mice, a model of acute-on-chronic alcohol consumption not previously used to study neuroinflammation, induced expression of multiple proinflammatory cytokine genes including $\text{Tnfa, Ccl2, Hmgb1, Il-17}$ and $\text{Il-23}$ in the brain cortex (Fig 2.9).

Previous studies indicate that antibiotic treatment that reduces intestinal bacterial load also reduces alcohol-induced inflammation in the liver\textsuperscript{72}. Here, we hypothesized that translocation of gut bacterial products to the CNS contributes to alcohol-induced neuroinflammation and that this process is regulated by the gut microbial load. Therefore, we sought to investigate whether gut decontamination using antibiotics could protect from neuroinflammation associated with alcohol consumption. We observed that proinflammatory cytokine expression was significantly increased in the cortex in alcohol-fed mice compared to pair-fed controls; however, this was markedly reduced in mice treated with antibiotics (Fig 2.9). The cerebral cortex has previously been identified as a site of alcohol-induced microglial activation, astrogliosis and proinflammatory cytokine increases\textsuperscript{269}. Antibiotic treatment fully prevented alcohol-related induction of $\text{Ccl2, Il-17}$ and $\text{Il-23}$ mRNA expression in the cortex. $\text{Tnfa}$ was induced in antibiotic-treated, alcohol-fed mice compared to antibiotic-treated pair-fed mice but its expression was still significantly lower compared to alcohol-fed mice without antibiotic treatment.

Expression of $\text{Tnfa, Ccl2, Il-17}$ and $\text{Il-23}$ was also reduced in the cortex of antibiotic-
treated pair-fed mice compared to those without antibiotic treatment. These results indicate that acute-on-chronic alcohol feeding in mice increases proinflammatory cytokine induction that is prevented by antibiotic treatment and reduced gut microbiome.
Figure 2.9 Antibiotic treatment protects from alcohol-induced inflammatory cytokine expression in the cortex. Expression levels of proinflammatory cytokines \( \text{Tnfa}, \text{Ccl2}, \text{Hmgb1}, \text{Il}-17 \) and \( \text{Il}-23 \) were measured from the cortex of pair-fed (PF) or alcohol-fed (EtOH) mice with or without daily antibiotic treatment (Abx). Data are mean +/- SEM, \( n=5-10 \) mice/group. \( *p < 0.05 \); n.s., not significant by Mann-Whitney test.
Cortical expression of inflammasome components increases with bacterial decontamination

Because we found that multiple proinflammatory cytokines were reduced in the cortex of antibiotic-treated mice (Fig 2.9), we next measured inflammasome-related transcripts to elucidate if alcohol or antibiotics influenced CNS inflammasome-mediated cytokine expression. The inflammasome is a multiprotein complex containing: NOD-like receptors (NLRs, including NLRP3) that can sense pathogens and danger signals; an adaptor molecule, ASC; and an effector molecule, caspase-1. Inflammasome activation leads to cleavage of pro-IL-1β and pro-IL-18 to their respective bioactive forms, IL-1β and IL-18162. Interestingly, although chronic alcohol consumption models have led to increased expression of inflammasome components and Il-1β162, we found no significant increase in Il-1β mRNA expression in this acute-on-chronic alcohol model (Fig 2.10). However, expression of Il-18 was induced in alcohol-fed mice in the cortex. While we did not observe an increase in Il-1β between PF and EtOH-fed mice without antibiotics, we did observe significantly elevated cortical Il-1β mRNA expression in antibiotic-treated pair-fed mice and an increasing trend in Il-1β in antibiotic-treated alcohol-fed mice. Interestingly, in antibiotic-treated mice, alcohol administration significantly increased Il-1β mRNA expression compared to pair-fed mice. Similar to the increase in pair-fed Il-1β, we also found that Il-18 and Asc were elevated in antibiotic-treated, pair-fed mice compared to untreated pair-fed mice. Acute-on-chronic alcohol administration reduced the expression of Nl rp3 and Asc and increased the expression of Il-18 in untreated alcohol-fed mice compared to untreated pair-fed controls. Asc and Il-18 mRNA
expression were reduced in antibiotic-treated compared to untreated alcohol-fed mice. 

Caspase-1 mRNA levels did not change significantly in any of the treatment groups (Fig 2.10). These observations suggest that regulation of the inflammasome and IL-1β depends on the gut microbiome and is minimally influenced in the acute-on-chronic alcohol model in mice.
Figure 2.10. The expression levels of inflammasome components, *Il-1β* and *Il-18* are increased in the cortex after antibiotic decontamination. Cortical expression of the inflammasome components *Nlrp3*, *Asc* and *Casp1* as well as the cytokines *Il-1β* and *Il-18*, were measured from the brains of pair-fed (PF) or alcohol-fed (EtOH) mice with or without daily antibiotic treatment (Abx). Data are mean +/- SEM, *n*=5-10 mice/group. *p* < 0.05; n.s., not significant by Mann-Whitney test.
Antibiotic treatment alters the morphology and IBA1 positivity of cortical microglia

To characterize the effects of the acute-on-chronic alcohol model in the CNS, we next examined microglia activation. Microglia are the resident macrophages of the CNS capable of expressing proinflammatory cytokines in response to an insult, such as alcohol. Activated microglia are characterized by altered cell morphology, taking on an amoeboid shape with enlarged cell bodies (soma) and shortened peripheral processes. We used immunohistochemistry to identify IBA1-positive microglia. The soma size was measured in these cortical microglia in all treatment groups and normalized to PF mice. No significant differences in soma size were observed between groups. Next, we measured the lengths of cell extensions coming off the soma and summed those measurements for a total cell process length. We found that alcohol reduced the total process length compared to pair-fed mice, consistent with the condensed cell morphology characteristic of microglial activation. Antibiotic treatment eliminated this alcohol-induced reduction in process length in the microglia. We measured total cortex IBA1 positivity and observed no difference between pair-fed and alcohol-fed mice. In contrast, IBA1 expression was markedly reduced in the cortex of antibiotic-treated mice in the presence and absence of alcohol administration. These results suggested that acute-on-chronic alcohol feeding results in morphologic changes in microglia that are prevented by the antibiotic-induced reduction in the gut microbiome.
Figure 2.11 Antibiotic treatment prevents alcohol-induced morphological changes in cortical microglia. A) Microglia were immunohistochemically stained for IBA1 and visualized at 40X magnification in the cortex of pair-fed (PF) or alcohol-fed (EtOH) mice. Representative microglia from the insets are shown in (B). C-D) Soma area (C) and cell process length (D) were measured in cortical microglia and normalized to the respective PF controls. E) Quantification of overall IBA1-positive staining area. Data are mean +/- SEM, n=3 mice/group and 5-9 images/region. *p < 0.05; n.s., not significant by Mann-Whitney test.
Alcohol-induced microglia changes in the hippocampus are normalized by antibiotic treatment

The hippocampus is a critical area of the brain associated with memory formation as well as addiction\textsuperscript{271}. Previous research has indicated that this brain region plays an important role in alcohol-associated pathology and is the site of significant neuroinflammation after alcohol consumption\textsuperscript{142}. Some patients with AUD suffer from Korsakoff syndrome, a significant memory disorder caused by alcohol-induced neurotoxicity and vitamin deficiency\textsuperscript{133} and various studies have identified the hippocampus, critical for learning and memory, as a key brain region impacted by alcohol exposure\textsuperscript{132,135}. Therefore, we sought to investigate the morphology of microglia in the hippocampus after acute-on-chronic alcohol feeding as morphological changes have previously been associated with neuroinflammation\textsuperscript{252,270}. IBA1-positive microglia were observed throughout the hippocampus (with representative dentate gyrus microglia displayed) (\textbf{Fig 2.12A-B}). Investigation of the sub-regions of the hippocampus, such as the CA1, CA3 and dentate gyrus (DG) areas, revealed that alcohol increased the soma area only in microglia of the CA3 region. There was no change in the soma area for CA3 microglia in alcohol-fed mice compared to pair-fed controls when treated with antibiotics (\textbf{Fig 2.12C}). The length of processes on the hippocampal microglia from alcohol-fed mice were significantly reduced compared to pair-fed controls in all regions investigated and, as in the cortex, antibiotic treatment eliminated this morphological change (\textbf{Fig 2.12D}). Alcohol increased the IBA1-positive area in the hippocampus and this was prevented by antibiotic treatment (\textbf{Fig 2.12E}). Similar to our observations in the cortex,
the total IBA1-positive area was decreased in the hippocampus by antibiotic treatment in both pair-fed and alcohol-fed mice (Fig 2.12E).
Figure 2.12 Hippocampal microglia morphology in CA1, CA3 and dentate gyrus is restored in antibiotic-treated alcohol-fed mice. A-B) Hippocampal microglia were immunohistochemically stained for IBA1 in pair-fed (PF) or alcohol-fed (EtOH) mice and visualized at 40X magnification with representative microglia from the insets in (B). C-D) Microglia soma area (C) and cell process length (D) in the CA1, CA3 and dentate gyrus (DG) of the hippocampus (HIPP). Data from alcohol-fed mice were normalized to the respective PF control. E) Quantification of overall IBA1-positive staining. Data are mean +/- SEM, n=3 mice/group and 5-9 images/region. *p < 0.05; n.s., not significant by Mann-Whitney test.
Discussion

This study provides, for the first time, insight into the microbiome changes that occur in a mouse model of early acute alcoholic steatohepatitis and neuroinflammation. The model does not induce differences in the $\alpha$-diversity or the $\beta$-diversity between intestinal bacterial communities in alcohol and pair-fed mice. However specific taxonomic shifts did reflect important changes in the makeup of the bacterial community in alcohol-fed mice and represent early indicators of developing alcoholic liver disease. Specifically, we identify *Akkermansia* decrease as an early change in alcohol-induced intestinal dysbiosis.

Our study utilized the acute-on-chronic feeding in which alcohol exposure spans a total of ten days. Others have investigated microbiome shifts in long-term alcohol feeding paradigms and observed $\alpha$- and $\beta$-diversity changes at later time points. In one such study, diversity measurements were increased in alcohol-fed mice after six-weeks of alcohol, while earlier time points were not investigated. That same study also showed trends in stool samples similar to ours, including a modest increase in the phylum *Actinobacteria* after alcohol administration. These similarities are particularly interesting because that study analyzed bacterial content of the stool while we examined bacteria from cecal content. In a short alcohol exposure model using seven-day intragastric alcohol infusion, Wang et al. described an increase in $\beta$-diversity between alcohol-infused and control mice. However, the intragastric infusion model varies significantly from the orally consumed Lieber-DeCarli liquid diet used in our study and the consumption method alone could explain the discrepant diversity findings.
In analysis of cecal content, we observed various taxonomic changes between alcohol- and pair-fed mice. Among these changes, phylum *Verrucomicrobia* was dominated by *Akkermansia* and its abundance was dramatically reduced by alcohol consumption. This finding is corroborated by careful investigation of other studies\textsuperscript{45} and offers a promising look into the potential importance of this genus in gut barrier maintenance disrupted by alcohol. Indeed, *Akkermansia* has been found to be decreased in mouse models of obesity and type-2 diabetes, while supplementation with the bacteria alleviated the burden of metabolic dysfunction\textsuperscript{272}.

Our data support previous studies showing that alcohol can induce inflammatory signaling in the intestine. This inflammation may be a key factor in the breakdown of the intestinal barrier integrity and ensuing leakage of bacterial products into the circulation associated with alcohol. Using both *in vitro* and *in vivo* models, Al-Sadi et al. have shown that proinflammatory cytokines are capable of reducing tight junctions and gut barrier integrity, leading to breakdown and molecule translocation across the gastrointestinal tract\textsuperscript{39-41}. Other mechanisms of alcohol-induced loss of intestinal barrier integrity have been explored and include bacterial dysbiosis\textsuperscript{45}, luminal homeostasis\textsuperscript{32,44} and enterocyte cellular stress and dysregulation of structural proteins\textsuperscript{37}. Furthermore, the relationship between proinflammatory gene expression and gut barrier dysfunction appears to be critical\textsuperscript{43,266}, and our data further emphasize the role of alcohol and intestinal bacteria in regulating intestinal cytokine levels. In the present study, we did not observe a statistically significant increase in serum LPS (one of many measures for intestinal barrier leakiness), suggesting that this model of alcohol feeding may not induce as robust
intestinal barrier leakage as other models. Commercially available LPS quantification assays require extensive optimization as there are many inhibitors and activators within samples leading to significant noise\textsuperscript{273,274}. Therefore, measuring alternative indicators of intestinal leakage (bacterial 16S rDNA in circulation, culturable bacteria from mesenteric lymph nodes, other bacterial surface biomarkers) could shed light on the state of the gut barrier. Additionally, further investigation into intestinal molecular changes (ex, tight junction protein expression), histologic examination of the intestinal barrier and into alterations in the cellular composition (immune cell within the lamina propria, support cells in intestinal crypts, etc.) in this model of alcohol administration, as has been explored in other models of chronic alcohol\textsuperscript{32,50}, will be informative.

Beyond the effects of alcohol on bacterial community changes, we also investigated the role of overall bacterial load on hallmarks of steatohepatitis showing that gut bacterial load reduction attenuated immune cell infiltration, steatotic accumulation and inflammation in the liver. Intestinal microbiota and bacterial products are involved in the pathogenesis of alcoholic liver disease\textsuperscript{21,22}. Infiltration of immune cells, especially neutrophils, is an important component of the acute-on-chronic model\textsuperscript{110,275}. However, the question of whether neutrophils are brought in to repair damage or whether their presence causes damage in the liver remains unclear. Here, we reduced the hepatic infiltration of neutrophils by treating with antibiotics without protecting the liver from damage (indicated by increased serum ALT and AST). This is partially corroborated by Bertola et al. who used an anti-neutrophil (anti-Ly6G) antibody and showed near complete elimination of neutrophil infiltration into the liver, but only partial protection
from transaminase increase. A possible explanation for the continued elevation of ALT and AST, despite protecting the liver from infiltrating inflammatory cells such as neutrophils, comes from the nature of the feeding model. This acute-on-chronic model includes an oral gavage of high-dose ethanol after ten days of feeding. The metabolism of this large dose of alcohol in a short time may induce hepatocyte damage and transaminase release regardless of the presence or absence of other damage or pathogen-associated signals. Suppression of circulating bacterial components (such as LPS) reduces the inflammatory signaling in the liver that would normally attract immune cells such as neutrophils to the site of liver injury.

We describe the protective role of antibiotic-mediated reduction in bacterial load in alcohol-induced inflammatory signaling, steatotic changes and neutrophil infiltration in the liver. Reducing circulating bacterial products, such as LPS (Fig 2.3B), likely reduces signaling via TLR4 and subsequent inflammatory signaling. Additionally, the reduction in circulating CCL2 protein in antibiotic-treated mice may contribute to the protection from alcohol-induced steatosis we report here, as we have previously shown the role of CCL2 in promoting lipid accumulation. We describe a protective role of antibiotic treatment in reducing circulating CCL2, neutrophil infiltration and steatotic changes within the liver. This protection occurs without the protection of ALT and AST increases, indicative of hepatocyte damage, suggesting that this protection is related to a role of the antibiotics independent of primary protection of the main parenchymal cell in the liver. Antibiotics reduced the intestinal bacterial load as well as circulating LPS which activates multiple immune process. Further study is needed to confirm whether
liver steatosis and inflammatory signaling is truly independent of primary hepatocyte damage and could include the use of immune cell-specific knockouts to inhibit immune activation (such as TLR4, the main LPS receptor) or perhaps protecting hepatocytes from alcohol-induced damage by knocking out enzymes involved in alcohol metabolism. Identifying whether immune and steatotic changes persists in the liver despite the absence of alcohol metabolism (and the cellular damage associated with its byproducts) could shed light on this important question.

To further investigate the effect of alcohol and bacterial reduction on steatosis, we measured mRNA levels of liver transcripts related to fat metabolism and lipid regulation. Interestingly, we found that ethanol reduced expression of fatty acid synthase (Fasn), uncoupling protein-1 (Ucp1), PR-domain zinc finger protein 16 (Prdm16), stearoyl-CoA desaturase 2 (Scd2). Antibiotic treatment also reduced expression of these genes compared to pair-fed mice without antibiotics. Gene suppression by antibiotics is consistent with other studies that have shown antibiotic treatment can have a negative impact on fat accumulation in the liver277.

Adipose differentiation-related protein (Ardp) and lipocalin-2 (Lcn2) are both involved in storage and transport of accumulated lipids within the cell. Their increased expression (Lcn2 p=0.10) in alcohol-fed animals compared with pair-feds, irrespective of antibiotic treatment, mirrors the increased lipid store within cells observed on histology. Therefore, we have observed that genes regulating lipid production tend to be suppressed by antibiotics and alcohol in this feeding model, while genes associated with regulating...
lipid stores (Adrp, Lcn2) are elevated presumably to compensate for the alcohol-induced fat accumulation.

Many of the liver findings intriguingly contradict those observed in germ-free mice\textsuperscript{55} which displayed significantly greater liver injury, inflammation and steatosis when compared with conventional mice in a model of acute alcohol exposure. In that study, germ-free mice were given a single gavage of 3g/kg ethanol and sacrificed 9h later (compared to our treatment of ten days of 5\% EtOH in liquid diet and a final gavage of 5g/kg sacrificed 9h later). The lower alcohol dose of 3g/kg induced only modest changes in serum ALT, steatosis and inflammatory cytokine expression in alcohol- versus pair-fed conventional (bacteria-competent) mice. Additionally, while we successfully reduced gut bacterial load using antibiotic treatment, some intestinal microbes still persisted (Fig 2.3E-F). It is possible that the remaining bacterial composition contained protective species (although we could not sequence these populations due to the low yield of bacterial DNA) or that the remaining bacteria may serve a homeostatic function in either intestinal metabolism or barrier maintenance\textsuperscript{54}.

In our study, we observe changes in the liver, including inflammatory cytokine expression, elevated markers of liver injury and immune cell infiltration in the alcohol-fed mice, despite relatively stable communities of gut bacteria (i.e. no dramatic shifts in diversity) compared to long-term alcohol feeding\textsuperscript{49}. Long-term alcohol feeding (6- to 8-weeks) induces both shifts in alpha- and beta-diversity as well as changes at the phylum level and other taxa, including significant increases in the phylum \textit{Firmicutes} and reduction in phylum \textit{Bacteroidetes}\textsuperscript{49}. This suggests that while some features of alcoholic
liver disease may be related to shifts in the gut bacterial composition seen over the long-term, there are other features, such as inflammation signaling and immune cell infiltration and hepatocyte injury that may be independent of bacterial diversity changes.

In the present study, we found evidence of microglial activation by acute-on-chronic alcohol administration in mice. Activated microglia take on an amoeboid-like morphology with reduced process length and, typically, an increased soma size. We found that average cell process length was decreased in alcohol-fed mice in addition to CNS proinflammatory cytokine expression increases, indicative of a neuroinflammatory state. Acute-on-chronic alcohol reduced cell process length in both the cortex and hippocampus and significantly increased the soma size in the CA3, a region of the hippocampus important for spatial learning and short-term memory.

Similar to observations in the liver, antibiotic treatment protected the CNS from proinflammatory gene expression and changes in the resident macrophage population induced by alcohol consumption. As already discussed, germ-free mice do not show the same protection from alcohol-induced liver damage that we describe using antibiotic decontamination. While studies in germ-free mice focused on CNS inflammation are lacking, recent reports suggest that the microbiome plays a critical role in regulating microglial maturation and function. Germ-free mice have dysregulated microglia with blunted immune reactivity, display immature phenotypes and are altered in distribution and numbers. Additionally, mice treated with long-term (four weeks) broad-spectrum antibiotics had microglia that displayed immature morphology. Replenishing germ-free mice with a complex microbiota or treatment with short-chain fatty acids served to
partially restore microglial morphology\textsuperscript{278}. Our present study used a shorter course of antibiotic treatment (ten days versus four weeks) in addition to alcohol feeding and changes in microglia morphology (cell processes) were observed in alcohol-fed mice. Further studies using germ-free animals and alcohol feeding will be beneficial in understanding the contributions of the microbiome and microglia to neuroinflammation. Additionally, selectively replenishing germ-free mice with a microbiome rich in particular bacterial communities, such as \textit{Akkermansia} and other “beneficial” species, accompanied by alcohol treatment, will shed important light on the connections between the microbiome, microglia and neuroinflammation. These future studies will be of additional interest because anti-inflammatory antibiotics such as the tetracyclines have been shown to reduce alcohol consumption and seizures following alcohol exposure in mice\textsuperscript{239,260}. Whether or not these behavioral observations are due to the antibiotic effect on the microbiome, on microglia or elsewhere in the CNS deserves further study to explore yet unknown connections between the microbiome and alcohol drinking behavior.

Our data are consistent with previous studies examining the role of TLR4 signaling in alcohol-related organ pathology. While some have suggested that alcohol may interact directly with TLR4 or affect lipid membrane interactions required for proper TLR4 signal transduction\textsuperscript{187,279}, TLR4 also recognizes endogenous (including HMGB1)\textsuperscript{168,169} and exogenous (PAMPs such as LPS)\textsuperscript{167} danger signals. Studies show that TLR4 knockout and knockdown mice are protected from numerous inflammation-related sequelae of alcohol exposure in the liver\textsuperscript{280} and in the brain\textsuperscript{170-172}. Rather than focusing on
TLR4 and its signaling pathway, we used antibiotics to reduce bacterial LPS, one of the prominent ligands of TLR4, and reveal a similar reduction in tissue inflammation from the gut to the brain. Our study adds critical evidence to the understanding of the gut-brain axis that relates multifocal pathology in the body after chronic alcohol exposure.

An important remaining question is whether gut bacteria or their products are primarily responsible for organ damage. A direct link between LPS and organ inflammation is possible: leakage of live or dead bacteria or bacterial-derived products into the systemic circulation has been documented in various alcohol administration settings\textsuperscript{24,26,31,32}. These bacterial signals could be directly responsible for inducing inflammation in the gut, liver and in the brain, as well as the associated organ damage. Although LPS does not cross the blood-brain barrier at significant levels\textsuperscript{173}, it could be interacting with juxta-cerebrovascular cells to transmit an immune signal across the barrier. Evidence of blood-brain barrier disruption in alcohol models and human patients provides another explanation for a possible direct mechanism of LPS-induced neuroinflammation\textsuperscript{174}. LPS may also act by inducing peripheral inflammation and expression of cytokines that can easily cross the blood-brain barrier. Regardless of how LPS may be communicating with the CNS, peripheral LPS exposure induces neuroinflammation including astrogliosis, microglial activation and cytokine signaling\textsuperscript{252}.

Alternatively, gut-derived signals, such as LPS, bacterial metabolites or other undescribed intestinal signals, could lead to a systemic reaction. This reaction could include inflammatory cytokines or activated immune cells in the liver or in circulation that then induce organ-specific inflammation in the CNS and elsewhere in the body.
Developing models to study this question will be a critical area of further study to explain inter-organ communication after alcohol consumption.

Interestingly, components of the inflammasome were affected both by antibiotic decontamination as well as alcohol consumption in the intestine and in the brain in our experiments. Unlike the effect on other proinflammatory cytokines, gut decontamination generally induced an increase in the expression of inflammasome components in the intestine and cortex, while acute-on-chronic alcohol had limited effects on expression. Recent studies have revealed that the inflammasome and gut microbiota interact in important ways, including inflammasome-dependent remodeling of the flora to protect from autoimmunity\textsuperscript{268}. Inflammasome activation requires two signals to first induce expression of effector molecules like pro-IL-1β (typically through TLR4 signaling) and a second signal to activate the inflammasome cascade leading to pro-caspase-1 and pro-IL-1β cleavage. Alcohol, which induced intestinal $\text{Il-1β}$ expression (encoding uncleaved pro-IL-1β), has been previously shown to reduce certain bacterial phyla, including \textit{Verrucomicrobia}\textsuperscript{72,281}, which is also decreased in mice with overactive NLRP3 inflammasome\textsuperscript{268}. Restoration of \textit{Akkermansia}, a bacterial genus within the \textit{Verrucomicrobia} phylum, was shown to be protective in liver disease\textsuperscript{282}, including alcohol-related pathology\textsuperscript{281}, suggesting a strong connection between alcohol, the inflammasome and gut bacterial dysbiosis.
Conclusion

This study supports the hypothesis that the gut microbiota are impacted by alcohol consumption and that the acute-on-chronic feeding model alters the microflora at multiple taxonomic levels. We conclude that gut microbes influence intestinal, liver and CNS inflammation, neutrophil infiltration and liver steatosis following alcohol consumption and these data further emphasize the gut-liver-brain axis, even in early alcohol-induced inflammation.
CHAPTER III

Pharmacological inhibition of CCR2/5 signaling prevents and reverses alcohol-induced liver damage, steatosis and inflammation in mice

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Abstract

Kupffer cell (KC) and macrophage activation contributes to steatosis, inflammation and fibrosis in alcoholic liver disease (ALD). We found increases in macrophages as well as T cells in the livers of human alcoholics, along with increased expression of Ccr2 and Ccr5 and their corresponding chemokines, CCL2 and CCL5. We hypothesized that inhibition of CCL2 (MCP-1) signaling with the dual CCR2/5 inhibitor, Cenicriviroc (CVC), would attenuate ALD. In a mouse model of ALD, liver injury (ALT) and steatosis were prevented by CVC whether used as “prevention” throughout the alcohol feeding or as “treatment” started after the development of ALD. Alcohol-induced increases in liver fibrosis (Sirius Red, Collagen mRNA expression and hydroxyproline) were normalized by both “prevention” and “treatment” with CVC. We found that “prevention” and “treatment” with CVC reversed alcohol-related increases in liver cytokine mRNA expression, such as Tnfα, Il-1β, Il-6, Ccl2 as well as TNFα, IL-1β and IL-6 protein levels. Both CVC regimens prevented the increase in infiltrating macrophages (F4/80lo CD11bhi) in livers of alcohol-fed mice. CVC increased liver T cell numbers and attenuated Il-2 expression without an effect on CD69+ or CD25+ T cell expression. In vitro, CVC inhibited CCL2-induced increases in hepatocyte Fasn and Adrp while it augmented Acox-1, Pgc1α and Ucp-2 expression, suggesting mechanisms for attenuated hepatocyte steatosis after CVC treatment. Finally, we found that CCL2 and CCL5 sensitized hepatocytes to lipopolysaccharide-induced liver TNFα, ALT and LDH release and caspase-3 activation and that CVC prevented this injury. In summary, our
data provides preclinical evidence for CCR2/CCR5 inhibition with CVC as a potent intervention to ameliorate alcohol-induced steatohepatitis and liver damage.
Introduction

Alcoholic liver disease (ALD) is an increasing health concern worldwide. The clinical pathophysiology of ALD involves a progressive spectrum of steatosis, steatohepatitis, fibrosis, cirrhosis, and increased risk of hepatocellular carcinoma. While simple fatty liver, induced by binge alcohol consumption, can be reversed after alcohol cessation, more severe forms of liver injury can develop in up to 35% of heavy alcohol drinkers. There is no definitive treatment for progressive ALD, except for abstinence, and the current standard of care with prednisolone for acute alcoholic hepatitis has limited efficacy, many contraindications, and side effects. Thus, an urgent need to explore novel therapies for ALD is apparent.

The pathomechanism of ALD has been attributed to direct effects of alcohol and its metabolites inducing macrophage infiltration, steatosis, and hepatocyte cell death through increased cytokine/chemokine receptor signaling and proinflammatory cytokine induction in the liver. The C-C chemokine receptor types 2 and 5 (CCR2 and CCR5), which signal through G proteins and other intracellular molecules, and their respective ligands, C-C chemokine ligand types 2 (CCL2; also known as monocyte chemoattractant protein-1 [MCP-1]) and CCL5 (also known as regulated on activation, normal T cell expressed and secreted [RANTES]) are implicated in liver inflammation and macrophage recruitment to the liver. In response to animal models inducing hepatocyte injury, such as acetaminophen, carbon tetrachloride or bile duct ligation, resident liver macrophages (Kupffer cells) secrete CCL2 promoting monocyte recruitment and migration to the liver, where they mature into proinflammatory macrophages. CCR2 and CCR5 mediate the
intrahepatic immune-cell interactions that promote activation and migration of peripheral monocytes to the liver\textsuperscript{90,91}. Activation of inflammatory cells and upregulation of several soluble inflammatory mediators, including the ligands of these key receptors, CCL2 and CCL5, are features of ALD\textsuperscript{92}. In animal studies of ALD, mice with CCL2 deletion or pharmacological blocking of CCL2 showed protection\textsuperscript{93,94}. Similarly, animal models lacking CCL5 display lower immune-cell activation and reduced liver fibrosis\textsuperscript{95}. Therefore, CCR2 and CCR5 are promising targets for treatment of ALD.

Cenicriviroc (CVC) is a novel, oral, dual CCR2/CCR5 antagonist with nanomolar potency against both receptors, and a long plasma half-life (30–40h in humans)\textsuperscript{284}. Phase 2 studies 652-2-201 (NCT01092104) and 652-2-202 (NCT01338883), conducted in human immunodeficiency virus (HIV)-infected participants have validated CVC blockade of CCR2 and CCR5 with a reciprocal increase in CCL2\textsuperscript{284-286}. In addition, marked reduction in a circulating biomarker of monocyte activation, soluble cluster of differentiation 14 (sCD14), was observed with CVC treatment in HIV-infected adults (study 652-2-202) and in adults with NASH and liver fibrosis (652-2-203; CENTAUR; NCT02217475) over up to 48-52 weeks of therapy\textsuperscript{285,287}. Anti-inflammatory and anti-fibrotic effects of CVC have been demonstrated in multiple animal models of acute liver injury and fibrosis\textsuperscript{225,288-290}. A post hoc analysis of phase 2b clinical study 652-2-202 (NCT01338883) revealed reductions in the fibrosis indices AST to Platelet Ratio Index (ARI) and Fibrosis-4 (FIB-4), which were significantly correlated with decreases in sCD14 in HIV-infected subjects treated with CVC at 48 weeks\textsuperscript{291}. The efficacy, safety and pharmacokinetics of CVC in adults with NASH and liver fibrosis compared to
placebo have been evaluated in the 2-year CENTAUR study; results of the Year 1 primary analysis showed that CVC was well tolerated and resulted in twice as many participants achieving ≥1 stage improvement in fibrosis and no worsening of steatohepatitis versus placebo. CVC is currently undergoing Phase 3 evaluation for the treatment of liver fibrosis in adults with NASH (AURORA; NCT03028740). Therefore, CVC is an attractive candidate for the treatment of ALD through antagonism of CCR2/CCR5 receptors.

Here, we hypothesize that CCR2/5 blockade using CVC will inhibit macrophage infiltration, lower inflammation and reduce steatosis and thereby alleviate ALD. We employ the Lieber-DeCarli model of ALD and administer CVC throughout the alcohol regimen for 6 weeks (hereafter referred to as “prevention”) or for the final 3 weeks (“treatment”) of alcohol administration. Our results show that CVC treatment significantly improves the hallmark phenotypes of ALD including hepatocyte damage, macrophage infiltration and inflammation in the liver. Our novel in vivo findings presented here suggest that CCR2/5 antagonism using CVC is a potential therapy for ALD.
Methods

Human Alcoholic Liver Disease Samples

Alcoholic liver disease (ALD) samples were obtained from The Liver Tissue Cell Distribution System (LTCDS, Department of Pediatrics, University of Minnesota, Minneapolis, MN) and included ten human liver samples from patients with end-stage alcoholic cirrhosis who received liver transplantation. Alcoholic cirrhosis was diagnosed by LTCDS and was based on a history of alcohol drinking and liver histology. Control human liver tissue was the non-involved surrounding tissue obtained from patients undergoing partial hepatectomy for liver cancer. The protocol for using these liver samples has been approved by the Liver Tissue Cell Distribution System of the University of Minnesota and the Institutional Review Board (IRB) of the University of Massachusetts Medical School. An informed consent in writing was obtained from each patient and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the IRB. Peripheral blood mononuclear cells (PBMCs) were obtained from patients with alcoholic hepatitis at the University of Massachusetts or from healthy controls with no history of ALD as part of a study approved by the IRB of the University of Massachusetts Medical School.

Alcohol Feeding and CVC Administration

The study protocol was approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School. All the methods were carried out in accordance with the approved guidelines. Eight-week-old C57BL/6J mice
were divided into alcohol- and pair-fed (control) groups as well as two CVC alcohol-fed groups. Alcohol-fed mice received 5% alcohol in Lieber-DeCarli liquid diet *ad libitum* as previously described. Pair-fed animals received a calorie-matched liquid diet. Some alcohol-fed mice received daily subcutaneous injections of 15mg/kg BW CVC for six weeks (“prevention” cohort) while others received the same dose daily for three weeks (beginning at week four through six of alcohol feeding; “treatment” cohort). 10% hydroxypropyl-β-cyclodextrin, 5% solutol HS15 and 85% sterile water was used as a vehicle to dissolve CVC into solution. At sacrifice, blood and liver tissues were collected for further assays.

**Isolation of Hepatocytes and Liver Mononuclear Cells**

Liver cell isolations were performed as described previously. Mice received anesthesia with ketamine (100mg/kg BW) and xylazine (10mg/kg BW) and livers were perfused with HBSS followed by *in vivo* digestion with collagenase enzyme (900mg/L). Hepatocytes were separated by centrifugation for five minutes at 300rpm and the pellet was washed in HBSS containing 2% FBS and were plated on collagen-coated plates. The non-hepatocyte content was loaded on the top of the 40%-70% Percoll gradient and centrifuged for thirty minutes at 1800rpm. Hepatocytes The liver mononuclear cellular (LMNCs) fraction from the Percoll interface was washed and immediately stained for flow cytometry or lysed in Qiazol (Qiagen, Germantown, MD, USA).

**Flow Cytometry**
Isolated LMNCs were suspended at a concentration of $10^6$ cells/ml in PBS and stained with Live/Dead Fixable Blue Dead Cell Stain Kit from Life Technologies (Grand Island, NY, USA) to exclude dead cells. Anti-mouse CD16/CD32 mAb from BD Pharmingen (San Jose, CA, USA) was used to block nonspecific Fc receptor binding and incubated for 20 min at 4°C. LMNCs were then immunostained with anti-CD45-Pacific Blue, anti-CD11b-PE, and anti-F4/80-APC, anti-CD3-PE, anti-CD25-FITC or anti-CD69-APC from Biolegend (San Diego, CA, USA). Hepatocytes were stained with anti-CCR2-AF647 and anti-CCR5-PE from Biolegend and albumin (Abcam, Cambridge, MA, USA) with a donkey anti-rabbit Brilliant Violet 421 secondary from Biolegend. Live singlet CD45$^+$ cells were gated and differentiated based on expression of CD11b and F4/80 or CD3, while albumin$^+$ cells were selected after gating live single cells. As a negative control, cells were stained with isotype-matched control antibodies. Cells were washed with FACS staining buffer (2% FBS in PBS) and fixed with 1% paraformaldehyde prior to data acquisition. Data were acquired on a BD LSR II instrument (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo v10.1 software (Ashland, OR, USA).

**Biochemical Assays**

Human serum chemokines were measured by CCL2 and CCL5 ELISA kits (BD Biosciences, San Jose, CA, USA). Serum alanine aminotransferase (ALT) activity was determined using a kinetic method (D-TEK LLC, PA, USA). Intracellular cytokine levels were monitored in liver whole cell lysate using TNF$\alpha$, MCP-1, IL-1$\beta$ ELISA kits (Biolegend, San Diego, CA, USA). Liver triglycerides were assayed in liver whole cell
lysates using L-Type TriGlyceride M kit (Wako Diagnostics, Richmond, VA, USA). Mouse serum soluble CD14 was measured by ELISA (R&D, Minneapolis, MN, USA). LDH assay (Abcam) and caspase-3 assay (R&D Systems) were completed according to the manufacturers’ recommendation.

RNA Extraction and Real-Time PCR

Total RNA was extracted using the Direct-zol RNA MiniPrep according to the manufacturer’s instructions (Zymo Research, Irvin, CA, USA). RNA was quantified using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). Complementary DNA (cDNA) synthesis was performed by reverse transcription of total RNA using the iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Real-time quantitative PCR was performed using the CFX96 real-time detection system (Bio-Rad). Primers were synthesized by IDT, Inc. (Coralville, IA, USA) and are shown in Table 1. Accumulation of PCR products was detected by monitoring the increase in fluorescence of double-stranded DNA-binding dye SYBR Green during amplification. Relative gene expression was calculated by the comparative cycle threshold (Ct) method and the expression of target genes was normalized to the house-keeping gene (18S rRNA) in each sample and the fold-change in the target gene expressed compared to pair-fed values, as previously described294. Melt-curve analysis was used to confirm the authenticity of the PCR products.

Table 3.1 Real-time PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tbody>
<tr>
<td>hCcr2</td>
<td>ACTGGTCCCTTAGCCCCATCT</td>
<td>CATCGGACTCCACCCAAAAACT</td>
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The above forward and reverse sequences of primers were used in real-time PCR. The 18S primers recognize both the human and mouse genes.

**Histopathological Analysis**

Sections of formalin-fixed, paraffin-embedded livers were stained with hematoxylin and eosin (H&E) or Sirius Red. To assess steatosis, Oil Red O staining was performed on 12-14µm cryofrozen liver tissue sections. Immunohistochemistry staining was performed on formalin-fixed, paraffin-embedded livers using antibodies against

<table>
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<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
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<tr>
<td>hCcr5</td>
<td>GGTTGGTGTTGCAGAAAGTT</td>
<td>TGGTCTCCTTGCACCTTAAATG</td>
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<tr>
<td>hCd11b</td>
<td>ACTGGTGAAAGGAATAACGC</td>
<td>TCCGTGATGACAATCTAGGA</td>
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<td>hCd68</td>
<td>CTTCTCTACTCCCTATTG</td>
<td>GAAAGGACACATTGACTCCA</td>
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<tr>
<td>hCd14</td>
<td>ACGCCAGAACCTTGTAGC</td>
<td>GCATGGGATCCTCACTCAC</td>
</tr>
<tr>
<td>hCd3</td>
<td>TGCTGTGGTTTACTACTGGA</td>
<td>GGATGGGCTCATAGTCTGG</td>
</tr>
<tr>
<td>mCollagen</td>
<td>GCTCCTCTAGGGCCACT</td>
<td>CCACGTCTCACCATTG</td>
</tr>
<tr>
<td>mTnfα</td>
<td>GAAGTTCCAAATGGGCTCC</td>
<td>GTGAGGGTCTGGGCATAG</td>
</tr>
<tr>
<td>mIl-1β</td>
<td>CTTGGAAAGGTGACGAGCC</td>
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<tr>
<td>mIl-6</td>
<td>ACAACCAGGGCCTCCCTTA</td>
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<td>mCcl2</td>
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<td>CCCCCACATAGGGATCATG</td>
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<td>mCcr5</td>
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<td>TAGGCCACAGCATGCCATT</td>
</tr>
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<td>mFos</td>
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<td>mIl-2</td>
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<td>mIfnγ</td>
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<td>GTTGGCTGATGGCCTGATG</td>
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<td>mFas</td>
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<td>TGGGAATATCCATAGAGC</td>
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<td>mAcox-1</td>
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<td>mAdrp</td>
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<td>mUcp-2</td>
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<td>ATTGTGTCCGGGACAG</td>
</tr>
<tr>
<td>18S</td>
<td>GTAACCCGTGAACCCCATT</td>
<td>CCATCCAATCCTGATAGCG</td>
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CD11b, CD14, CD68 and CD3 (Abcam). Images were acquired on a Nikon microscope. The quantification of the Oil Red O and Sirius Red staining was performed using ImageJ software.

**In vitro Experiments**

For *in vitro* sensitization experiments, mouse primary hepatocytes were isolated as described above, plated on collagen-coated plates and allowed to rest for at least four hours. The media was changed and cells were then stimulated with either 200ng/mL CCL2 (MCP-1; PeproTech, Rocky Hill, NJ, USA) or 20ng/mL CCL5 (RANTES; PeproTech) for 24h. Some groups received either 10nM, 20nM or 100nM CVC. After changing the media, 10ng/mL lipopolysaccharide (LPS) was added for 18h after which culture supernatants were collected for measurement of TNFα, ALT and lactate dehydrogenase (LDH). Cell lysates were prepared for caspase-3 assay.

**Statistical Analysis**

Statistical significance was determined using either two-tailed t-test or ordinary one-way ANOVA with Tukey’s multiple comparison post-test to compare the means of multiple groups. Data are shown as mean +/- SEM and were considered statistically significant at $p<0.05$. GraphPad Prism 6.02 (GraphPad Software Inc., La Jolla, CA, USA) was used for analysis.
Results:

Patients with ALD have elevated expression of the CCR2/5 signaling axis and increased leukocyte infiltration in the liver

To investigate the clinical significance of CCR2/5 in ALD, we first confirmed the presence of these receptors in livers of human patients with alcoholic cirrhosis. Messenger RNA expression of Ccr2 and Ccr5 as well as their ligands Ccl2-5 and Ccl8 was significantly upregulated in livers of patients with alcoholic cirrhosis compared to control livers (Fig 3.1A). Peripheral blood mononuclear cells (PBMCs) from patients with alcoholic-hepatitis have increased expression of Ccl2 and Ccl3 and decreased Ccl5 compared to healthy control PBMCs (Fig 3.1B). CCL2 (or MCP-1) and CCL5 (or RANTES) are chemokines that bind to their respective receptors, CCR2 and CCR5, and activate downstream signaling. We evaluated the levels of the ligands of these receptors in serum samples obtained from patients with acute alcoholic hepatitis. Both serum CCL2 and CCL5 levels were significantly higher in patients with alcoholic hepatitis compared to healthy controls (Fig 3.1C).

Macrophages play an important role in inflammation and progression of the liver injury in alcoholic liver disease (ALD) and cirrhosis. Indeed, we found increased expression of macrophage markers in human liver samples (Fig 3.1D). The gene expression of characteristic macrophage markers including Cdl1b, Cdl68 and Cdl14 were significantly upregulated in the alcoholic cirrhotic livers compared to controls (Fig 3.1D). Further, we observed increased macrophage infiltration in the human alcoholic livers
indicated by immunostaining for the macrophage markers CD11b, CD68 and CD14 (Fig 3.1E).

CCL5 plays a significant role in activation and migration of T cells, particularly in viral infection\(^{296}\). We found an increase in expression of Cd3 mRNA (Fig 3.1D) as well as an increase in the number of CD3\(^+\) T cells by immunohistochemistry in the livers of patients with alcoholic cirrhosis (Fig 3.1E). Together, our data demonstrate increases in macrophages and T cells in human alcoholic livers, elevation of CCL2 and CCL5 serum levels and increased expression of their receptors, CCR2/5, in human alcoholic liver disease.
Figure 3.1 CCR2 and CCR5 expression and immune cell infiltration to the liver is increased in human patients with alcoholic liver disease. A-B) Expression of CCR2 and CCR5 receptor and ligand genes were measured by qPCR in livers of patients with alcoholic cirrhosis or healthy controls or from peripheral blood mononuclear cells (PBMCs) from patients with alcoholic hepatitis or healthy controls. C) CCL2, CCL3, CCL4 and CCL5, chemokine ligands of CCR2 and CCR5, were measured from the sera of healthy controls and alcoholic hepatitis (AH) patients by ELISA. D) Expression of macrophage markers *Cd11b, Cd68 and Cd14 and T cell marker *Cd3 were measured by qPCR in the livers of controls and patients with alcoholic cirrhosis. E) Macrophage markers CD11b, CD68 and CD14 and the T cells marker CD3 were stained by immunohistochemistry in liver tissue sections from controls and patients with alcoholic cirrhosis. * p<0.05 by student’s t-test
Inhibition of CCL2/CCL5 signaling with a dual CCR2/CCR5 antagonist alleviates chronic alcohol-induced liver injury in mice

Having confirmed the elevation of 
\textit{Ccr2} and 
\textit{Ccr5} expression in human alcoholic liver, we tested the efficacy of Cenicriviroc (CVC), a dual CCR2/5 antagonist in a mouse model of alcoholic liver disease that is reliable for preclinical testing of potential interventions\textsuperscript{112,297}. Administration of CVC for 6 weeks during alcohol administration in the “prevention” cohort showed significant protection from alcohol-induced ALT increases with significant attenuation at 2-4 weeks and normalization of ALT to the level of pair-fed controls at weeks 5-6 (Fig 3.2A). Furthermore, when CVC was started at week 4 of the alcohol feeding in the “treatment” cohort, we found rapid normalization of ALT levels that remained at the level of pair-fed mice for the final 3 weeks of the alcohol feeding (Fig 3.2A).

We found a significant improvement in liver pathology in both “prevention” and “treatment” administration of CVC in ALD in mice (Fig 3.2B). Liver steatosis, estimated by Oil Red O staining was significantly lower after both regimens of CVC administrations compared to alcohol-fed without CVC treatment (Fig 3.2C). Liver triglyceride measurement further confirmed the inhibitory effect of CVC on alcohol-induced liver steatosis (Fig 3.2E). Chronic alcohol exposure leads to liver fibrosis in mice and often to cirrhosis in humans. Liver fibrosis was increased by alcohol feeding as assessed using Sirius Red staining (Fig 3.2D), Collagen mRNA expression in the liver (Fig 3.2F) and liver hydroxyproline (Fig 3.2G). We found a significant reduction in alcohol-induced liver fibrosis in CVC-treated mice both in the “prevention” and
“treatment” cohorts (Fig 3.2D-G). These results indicate that inhibition of CCR2/5 signaling protected mice from alcohol-induced increase in parenchymal injury and steatosis, improved liver pathology and restricted fibrosis in mice.
Figure 3.2 Inhibition of CCR2/5 signaling alleviates chronic alcohol-induced liver injury. Mice were fed a liquid diet containing 5% ethanol (Alc) or pair-fed (PF) diet for 6 weeks. Some mice were injected subcutaneously with the CCR2/5 inhibitor Cenicriviroc (CVC) daily for the entire 6 weeks or during the final 3 weeks (weeks 4-6) of ethanol feeding. Control mice were injected with vehicle. A) Serum ALT was measured weekly to track liver damage caused by chronic alcohol consumption. The Prevention Cohort received CVC daily beginning at the start of ethanol feeding while the Treatment Cohort began receiving injections at week 4. Data from PF + Vehicle and Alcohol + Vehicle are replotted on each graph to allow comparison with each administration group. n=3-4 mice/group for weeks 0-5 and 13-15 mice/group for week 6. * p<0.05 between Alcohol + Vehicle and Alcohol + CVC. B) Livers from pair-fed (PF), alcohol-fed or alcohol-fed plus CVC treated mice were stained with hematoxylin and eosin. C) Oil Red O staining was completed on mouse livers to visualize and quantify lipid accumulation. D) Sirius Red staining was completed to visualize and quantify fibrosis development. E) Liver triglycerides were measured. F) Liver expression of collagen mRNA was measured by qPCR. G) Liver hydroxyproline, a surrogate marker of collagen content, was measured using a colorimetric assay. * p<0.05 by one-way ANOVA.
CVC reduces macrophage infiltration to the liver in ALD

Macrophages are the primary contributors to alcohol-induced inflammation in the liver\textsuperscript{283}. Circulating monocytes respond to CCL2 chemoattraction via the receptor CCR2. We found that expression of $\text{Ccr2}$ mRNA by qPCR (Fig 3.3A) and F4/80\textsuperscript{+} cells, measured by immunohistochemistry (Fig 3.3B), were significantly increased in the livers after alcohol feeding compared to pair-fed controls. Furthermore, the alcohol-induced increase in $\text{Ccr2}$ mRNA and F4/80\textsuperscript{+} cell number were dramatically normalized to the level of pair-fed controls by CVC “prevention” or “treatment” (Fig 3.3A-B).

To examine the state of resident and infiltrating macrophages in the liver following alcohol consumption, we analyzed the CD45\textsuperscript{+}F4/80\textsuperscript{+}CD11b\textsuperscript{+} population of liver mononuclear cells\textsuperscript{283} (Fig 3.3C). We detected no significant difference in the total liver CD45\textsuperscript{+}F4/80\textsuperscript{+}CD11b\textsuperscript{+} macrophage population (Fig 3.3D) and no changes in CD45\textsuperscript{+}F4/80\textsuperscript{hi}CD11b\textsuperscript{lo} liver resident Kupffer cells (Fig 3.3E). However, we observed a robust increase in the number of CD45\textsuperscript{+}F4/80\textsuperscript{lo}CD11b\textsuperscript{hi} infiltrating liver macrophages after alcohol feeding that was abrogated both in the “treatment” and “prevention” CVC treated mice (Fig 3.3F). Soluble CD14 (sCD14) serum protein level, an indicator of monocyte activation, was significantly increased after alcohol treatment and reduced after CVC administration in both administration cohorts (Fig 3.3G).
Figure 3.3 CVC decreases the infiltration of peripheral macrophages into the liver. A) Liver mRNA expression of Ccr2, a target of CVC, was measured by qPCR. B) Liver sections were formalin fixed and stained by immunohistochemistry for macrophage surface marker F4/80 to identify hepatic macrophages. C-F) Flow cytometry was used to quantify total liver macrophages (D; F4/80<sup>+</sup>CD11b<sup>+</sup>), resident Kupffer cells (E; F4/80<sup>hi</sup>CD11b<sup>lo</sup>) as well as infiltrating liver macrophages (F; F4/80<sup>lo</sup>CD11b<sup>hi</sup>). Cells were gated based on size, singlets, dead cell exclusion using an amino-reactive dye and for CD45<sup>+</sup> positivity (C). * p<0.05 by one-way ANOVA.
CVC reduces proinflammatory cytokine production and macrophage activation in ALD

Proinflammatory cytokines produced by macrophages accelerate the progression of alcoholic liver disease\textsuperscript{298}. Administration of CVC significantly reduced liver mRNA expression of \textit{Tnfα, Il-1β, Il-6, Ccl2} (\textbf{Fig 3.4A}). The liver protein levels of TNFα, IL-1β and IL-6 were also significantly lowered by CVC, however, the tissue CCL2 protein levels did not change after CVC administration (\textbf{Fig 3.4B}), consistent with previous data in animal models suggesting that CCL2 mRNA expression in tissue did not change after CVC treatment\textsuperscript{288}. 
Figure 3.4 CVC inhibits pro-inflammatory cytokine expression induced by alcohol. A-B) Liver mRNA expression of pro-inflammatory cytokines Tnfa, Il-1β, Il-6 and Ccl2 were measured by qPCR (A) and the corresponding liver proteins were measured by ELISA (B). * p<0.05 by one-way ANOVA.
CVC blocks activation of T cells in alcoholic liver disease

CCL5 is induced in the liver upon alcohol consumption and CCR5 mediates the intracellular signaling cascade of CCL5. We next evaluated the effect of dual CCR2/5 inhibitor CVC on T cells in the liver. We found a significant increase in Ccr5 mRNA in livers of mice with alcoholic liver disease (Fig 3.5A). CVC significantly lowered Ccr5 expression in livers in both “prevention” and “treatment” cohorts (Fig 3.5A). Immunostaining for CD3 on mouse liver sections did not show significant differences after alcohol or CVC administration in either cohort (Fig 3.5B). FACS analysis of isolated liver leukocytes revealed that while alcohol consumption did not impact the proportion of CD3+ T cells in the liver, CVC administration surprisingly led to a significant increase in liver T cells (Fig 3.5C). Further flow cytometric analysis revealed that CD69+ activated T cells were not significantly altered by alcohol (Fig 3.5D), while CD25+ T cells were increased by alcohol consumption (Fig 3.5E). CVC in both administration methods tended to lead to reduced CD25 expression in T cells compared to alcohol alone (Fig 3.5E). Consistent with the increase in T cell activation (CD25 expression), Il-2 expression was increased by alcohol but attenuated by both CVC regimens (Fig 3.5F). Alcohol did not have an effect on expression of Foxp3, a key transcription factor of regulatory T cells, or Ifnγ expression (Fig 3.5F).
Figure 3.5 CVC reduces the infiltration and activation of T cells into the liver following chronic alcohol. A) Liver mRNA expression of Ccr5, another target of CVC, was measured by qPCR. B) Liver sections were formalin fixed and stained by immunohistochemistry for T cell surface receptor CD3 to identify hepatic T cells. C-E) Flow cytometry was used to quantify total liver T cells (C; CD3^+), activated CD69^+ T cells (D) as well as CD25^+ T cells (E). Cells were gated for lymphocytes, singlets, dead cell exclusion using an amino-reactive dye and for CD45^+CD3^+ positivity. F) Liver mononuclear cells were isolated via tissue digestion followed by density gradient and mRNA expression of T cell-associated genes Il-2, Foxp3 and Ifnγ were measured by qPCR. * p<0.05 by one-way ANOVA.
CVC blocks alcohol-induced steatosis in primary hepatocytes

Excess alcohol consumption leads to hepatic steatosis caused by the direct effects of alcohol and alcohol metabolites through modulation of hepatic fatty acid synthesis and reduction in fatty acid oxidation\(^{301,302}\) as well as through other mechanisms including CCL2 signaling. Consistent with previous reports that showed CCR2/5 expression on hepatocytes in hepatitis C virus-infected patients\(^{303,304}\), we confirmed by flow cytometry that primary hepatocytes in mice express CCR2/5 and their surface expression was upregulated after chronic alcohol exposure (Fig 3.6A). Next, we assessed the effect of CVC on fatty acid metabolism gene expression in primary hepatocytes in vitro. We found that CCL2-induced steatosis was attenuated by CVC at multiple levels: the fatty acid synthesis gene, Fasn, was down-regulated after CVC treatment in presence of CCL2 (Fig 3.6B), while the FA oxidation genes such as Acox-1, Pgc1\(\alpha\) were significantly upregulated after CVC treatment in vitro suggesting a direct effect on steatosis (Fig 3.6B).

CVC blocks alcohol-induced cell death in primary hepatocytes

While the robust effect of CVC on inflammation and macrophage recruitment can be explained by inhibition of CCR2 receptor signaling, the mechanism by which CVC normalized alcohol-induced hepatocyte injury is less clear. Thus, we hypothesized that CCR2/CCR5 inhibition may have direct protective effects on hepatocytes in ALD. Given the dramatic improvement of ALT levels after CVC administration (Fig. 3.2A) we sought to test if CCL2 or CCL5 signaling can initiate a hepatocyte response and if CVC can
protect against hepatocyte cell death. We found that CCL2 or CCL5 treatment resulted in sensitization of primary hepatocytes to LPS and resulted in TNFα release (Fig 3.6C-D). CCL2 or CCL5 treatment of primary hepatocyte followed by lipopolysaccharide (LPS) treatment also led to an increase in ALT and lactate dehydrogenase (LDH) release (Fig 3.6C-D). Importantly, all of these effects of CCL2 or CCL5 were inhibited by CVC in a dose-dependent manner (Fig 3.6C-D). Treatment with CVC lowered LDH and Caspase-3 activity and showed a significant reduction in TNFα production and ALT levels (Fig 3.6C-D). These results indicate that both CCL2 and CCL5 sensitize hepatocytes to LPS-induced injury and that CCR2/5 inhibition with CVC can prevent damage in primary mouse hepatocytes.
Figure 3.6 CVC protects from induction of steatosis, inflammation and cell death in primary hepatocytes. A) Expression of receptor proteins CCR2 and CCR5 was measured on primary mouse hepatocytes from animals fed alcohol or a calorie-matched diet. Both were elevated by chronic alcohol consumption. B) To assess the influence of signaling through CCR2, primary hepatocytes were isolated and cultured in vitro with either recombinant CCL2 alone, CVC alone or CCL2 with CVC. mRNA expression of genes associated with fatty acid metabolism revealed the effects of CCL2 and CVC. C-D) Either CCL2 (C) or CCL5 (D), ligands of CCR2 and CCR5 respectively, were used in combination with varying doses of LPS and CVC to observe the induction of TNFα as well as indicators of cell death including alanine aminotransferase (ALT), lactate dehydrogenase release and caspase-3 activity. CVC protected hepatocytes from damage in a dose-dependent manner. * p<0.05 by student’s t-test or by one-way ANOVA.
Discussion

Chronic inflammation, steatosis and hepatocyte injury are major components of the pathology and clinical manifestations of alcoholic liver disease\textsuperscript{106}. A hallmark of ALD inflammation is an increase in the chemokines CCL2 and CCL5 (MCP-1 and RANTES, respectively) which are capable of recruiting peripheral macrophages and T cells\textsuperscript{89}. The infiltration of circulating mononuclear cells (both macrophages and T cells) into the liver has been established as a feature of both animal and human alcoholic liver disease and is associated with disease severity\textsuperscript{283}. Our present study demonstrates that monocyte recruitment in particular through alcohol-induced CCR2/5 signaling contributes significantly to the phenotype of alcoholic liver disease including hepatocyte injury, development of steatosis and significant cytokine upregulation (\textbf{Fig 3.1-4}).

Hepatic protection was prominent using both a “prevention” dosing model, in which animals received daily CVC from the start of alcohol administration, as well as the “treatment” model where CVC began after ALD was already established (\textbf{Fig 3.2A}). Secretion of CCL2 leads to chemoattraction of macrophages by binding to the CCR2 receptor, while CCL5 chemoattracts T cells through CCR5\textsuperscript{89}. Blockade of the CCR2/5 receptors inhibits the characteristic alcohol-induced infiltration of peripheral macrophages as well as leukocyte activation markers (\textbf{Fig 3.3-5}). We found that CVC mitigates the metabolic shift in hepatocytes toward lipogenesis induced by CCL2 and reduces the damage induced by LPS, a gut-derived signal that causes significant liver inflammation and injury in alcoholic liver disease (\textbf{Fig 3.6}).
CCL2 is well known to be upregulated in hepatocytes as well as liver macrophages in alcoholic liver disease\textsuperscript{283}. Activation of TLR4 by the interaction with one of its ligands, such as gut-derived LPS in the setting of chronic alcohol, induces CCL2 expression which signals locally and chemoattracts peripheral cells\textsuperscript{305}. The infiltration of peripheral macrophages leads to significant damage in alcoholic hepatitis by contributing to fibrosis and cirrhosis\textsuperscript{306,307}. Circulating monocytes exhibit a proinflammatory phenotype in patients with alcoholic liver disease and consistent with this we found increased soluble CD14 levels after alcohol exposure that was attenuated by CVC administration. Peripheral monocytes also increase production of TNF\(\alpha\) and other cytokines, as well as increase responsiveness to LPS\textsuperscript{308-310}. Macrophage depletion has been shown to be effective at protecting from alcoholic liver disease in animal models\textsuperscript{112,311}. Here, we provide evidence for effective pharmacologic inhibition of alcohol-induced infiltration of macrophages into the liver in a mouse model of ALD. Macrophage expression of CCR2 is not binary and some circulating lymphocytes may infiltrate tissue using non-CCR2 receptor mediated methods\textsuperscript{312}. Our data suggests that blockade of CCR2/5 with CVC is sufficient to block alcohol-induced macrophage infiltration and support a CCR2-dependent mechanism for macrophage migration in chronic alcohol use.

T cells have long been implicated in the pathogenesis of alcoholic liver disease in which key chemokines, including CCL5, are upregulated in the liver\textsuperscript{313,314}. Here, we describe the upregulation of CCR5 in the livers of mice fed alcohol chronically. T cells were detected via both immunohistochemistry and flow cytometry in these livers,
however alcohol was not associated with an increase in T cell infiltration in this model. T cells of alcohol-fed mice have suppressed expression of CD62L (L-selectin), an important chemotactic protein, which may contribute to a trend toward the decreased liver T cells we observed. CD69 and CD25 transmembrane proteins are found on activated T cells. Interestingly, patients with alcoholic hepatitis have been found to have reduced numbers of circulating CD25+ T cells. In our study, we found that alcohol increased the number of CD25+ T cells within the liver where they may be homing to serve a particular function. We did not detect an increase in expression of the Treg transcription factor, Foxp3, in the liver mononuclear cell fraction, suggesting that the detected increase in CD25+CD3+ cells represented activated T cells. CVC administration did not statistically affect CD25 expression but did reduce IL-2 expression in both administration models. These data suggest that inhibition of CCR2/5 may be able to reduce activated T cells, consistent with reports that CCR5 may be involved in T cell activation in other disease states such as viral infection.

Interestingly, we have previously investigated the role of CCL2 and CCR2 in hepatocyte fatty acid oxidation. Using CCL2 and CCR2 KO mice, our group showed that induction of genes related to lipid metabolism was absent in CCL2 KO mice and was independent of CCR2 based on data from CCR2 null animals. In the present study, using a CCR2/5 dual inhibitor, we show both that steatosis as well as fatty acid synthesis gene induction can be reduced while fatty acid oxidation can be induced by blockade of the receptors. While CCR5 blockade is a new and a potentially beneficial component added in the present study, previous results do suggest the critical role played by CCL2 in liver
lipid regulation. CCR2 null mice did not exhibit protection from alcohol-induced steatotic changes indicating that perhaps reduced (but not absent) CCR2 signaling can serve a protective function, similar to the protection seen in CCL2 KO animals\textsuperscript{93}.

The applicability of these findings in the pre-clinical mouse model to human alcoholic liver disease and acute alcoholic hepatitis awaits future investigations. Based on observations in human acute alcoholic liver disease, where monocyte and macrophage activation are a major element of the disease process, inhibition of macrophage activation would be a feasible approach. While the role of T cells is yet to be fully understood in alcoholic hepatitis and ALD, dysfunctional T cells and T cell exhaustion have been described\textsuperscript{317}. Thus, dual CCR2/5 antagonism may have beneficial applications in the treatment of alcoholic liver disease.
CHAPTER IV

Chronic alcohol-induced neuroinflammation involves CCR2/5-dependent peripheral macrophage infiltration and altered synaptic maturation

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Writing: PL GS.

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Abstract

Chronic alcohol consumption is associated with neuroinflammation, neuronal damage and behavioral alterations including addiction. Alcohol-induced neuroinflammation is characterized by increased expression of proinflammatory cytokines (including TNFα, IL-1β and CCL2) and microglial activation. We hypothesized chronic alcohol consumption results in peripheral immune cell infiltration to the CNS. We further hypothesized that blockade of CCR2/5 signaling would inhibit alcohol-induced CNS infiltration of peripheral macrophages and would alter the neuroinflammatory state in the brain after chronic alcohol. Using CX3CR1<sup>GFP</sup>/+CCR2<sup>RFP</sup>/+ mice we found increased peripheral macrophage infiltration in the CNS, and particularly in the hippocampus, when mice were fed a chronic alcohol liquid diet compared to isocaloric pair-fed controls. Macrophage recruitment into tissue is mediated by CCL2 (MCP-1). Treatment with Cenicriviroc (CVC), a dual CCR2/5 inhibitor blocking CCL2 signaling, abrogated the alcohol-induced infiltration of peripheral macrophages. Chronic alcohol caused microglia to alter expression of surface activation markers and induced morphological changes consistent with an activated phenotype. Additionally, proinflammatory gene and protein expression was upregulated in various regions of the brain including the cortex, cerebellum and hippocampus and CVC treatment partially corrected these phenotypes. Finally, chronic alcohol significantly impacted synapses by inducing an increase in both excitatory (VGLUT1/HOMER1) and inhibitory (VGAT/Gephyrin) synapses in the hippocampus and these synapses were associated with increases in complement components C1q and C3, which are known to
signal synapses for pruning by microglia. Together these data indicate the role of peripherally-derived macrophages in alcohol-induced neuroinflammation and provide novel insights into the dysregulation of synapse maturation caused by chronic alcohol consumption.
Introduction

Alcohol use disorder (AUD) impacts millions of people across the world, including at least 17 million US patients\(^2\). Nearly 100,000 patients die each year because of AUD\(^2\), which induces organ injury throughout the body, including in the liver and in the brain. In both humans and mice, alcohol intoxication leads to central nervous system (CNS) inflammation and neurodegeneration\(^{12-14}\). Recent studies using animal models have shown that inflammatory signaling not only contributes to neurodegeneration but also to alcohol addiction\(^{15-17}\), making targeting of neuroinflammation a critical approach in the treatment of AUD.

Microglia, the resident macrophages of the CNS, become activated in the brain after alcohol use\(^{141,142}\). In addition to microglia, peripheral macrophages can be recruited into the CNS under pathologic conditions and may serve to amplify ongoing neuroinflammation\(^{200}\). Recent evidence suggests that breakdown of the blood brain barrier occurs in postmortem tissue of AUD patients and knockout of Toll-like receptor 4 (TLR4), an important innate immune signaling receptor expressed by microglia and peripheral macrophages, protects from the ensuing alcohol-induced neuroinflammation\(^{174}\). While microglial activation has been studied in alcohol-induced neuroinflammation, the potential infiltration of peripherally recruited macrophages is yet to be evaluated. Both infiltrating macrophages (IMs) and microglia become activated in response to tissue damage and can release proinflammatory cytokines, which may contribute to neuroinflammation and blood-brain barrier breakdown\(^{201}\).
In addition to their role in immune responses to tissue damage, recent research has highlighted an important function of microglia in shaping synapses in the CNS. Microglia respond to neuronal signaling and can engulf inactive or unnecessary synapses during normal physiological pruning. Synapses are marked for engulfment by members of the complement pathway which is a critical component of inflammation signaling in disease. Complement proteins C1q and C3 bind to synapses and are recognized by microglial complement receptors such as CR3. Perturbations in the CNS microenvironment, such as expression of cytokines that may activate microglia and impact homeostatic synaptic signaling, can have significant impact on proper synapse regulation and can result in dysfunctional neurodevelopment manifested in dramatic behavioral phenotypes. Indeed, binge alcohol models in rodents, which are known to activate microglia as well as various inflammatory pathways, have revealed altered synaptic development and pruning in the prefrontal cortex.

Peripheral macrophage chemotaxis through the receptor-ligand CCR2-CCL2 signaling axis is important in macrophage recruitment to the CNS. Recent studies have found an increase in the expression of the CCR2 ligand, CCL2 (also called monocyte chemoattractant protein 1; MCP1), in the brain after chronic alcohol consumption. Cenicriviroc (CVC) is a small molecule inhibitor recently developed to block signaling through CCR2, as well as the chemokine receptor CCR5 that recognizes CCL5 and is involved in recruitment of monocytes and T cells. CVC is currently under investigation in a Phase 3 trial to treat liver fibrosis in non-alcoholic steatohepatitis (NCT03028740), a disease that involves significant inflammation. CVC
has been shown previously to inhibit macrophage recruitment and activation in liver
disease\textsuperscript{225}.

We hypothesized that because CCL2 is highly expressed in human and mouse
brains after chronic alcohol use, peripheral IM recruitment is important in alcohol-
induced neuroinflammation and that treatment with CVC to inhibit infiltration of
peripheral macrophages would be neuroprotective. Here, we present evidence of region-
specific peripheral macrophage recruitment, associated with cytokine expression and
microglial activation after a model of chronic alcohol in mice. Treatment with CVC
reduced the number of IMs in the CNS, reduced cytokine expression and corrected
microglial morphology. Additionally, we report that chronic alcohol alters the number of
excitatory and inhibitory synapses in the hippocampus.

These data provide important insights into the role of IMs in alcohol-induced
neuroinflammation and offer a novel target for therapeutic treatment in this preclinical
model of alcohol use disorder.
Methods

Mice

The study protocol was approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School. All the methods were carried out in accordance with the approved guidelines. CX3CR1<sup>eGFP/eGFP</sup> mice were provided by Dr. Dorothy Schafer (Department of Neurobiology, University of Massachusetts Medical School). CCR2<sup>RFP/RFP</sup> mice were purchased from Jackson Labs. CX3CR1<sup>eGFP/wt</sup> CCR2<sup>RFP/wt</sup> mice were bred in-house. Wild-type mice were purchased from Jackson Labs and allowed to acclimate to our Animal Medicine facility for at least one week prior to experimental use. Eight-week-old female mice were used for alcohol feeding experiments. Eight-week-old C57BL/6J mice were divided into alcohol- and pair-fed (control) groups as well as two CVC alcohol-fed groups. Alcohol-fed mice received 5% alcohol in Lieber-DeCarli liquid diet <i>ad libitum</i> as previously described<sup>70</sup>. Pair-fed animals received a calorie-matched liquid diet.

CVC Administration

Some alcohol-fed mice received daily subcutaneous injections of 15mg/kg BW Cenicriviroc (CVC; provided by Tobira Therapeutics, Delaware, USA) for six weeks (“prevention” cohort) while others received the same dose daily for three weeks (beginning at week four through six of alcohol feeding; “treatment” cohort). 10% hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO USA), 5% Kolliphor (solutol) HS15 (Sigma) and 85% sterile water was used as a vehicle to dissolve CVC into solution.
Pair-fed mice and alcohol-fed mice received daily injections of the vehicle without CVC for six weeks.

**Brain Immune Cell Isolation**

Mice were anesthetized and transcardially perfused with PBS/heparin (Hospira, Lake Forest, IL, USA) to clear blood cells from the vasculature. Brains were dissected out and meninges removed. The tissue was then homogenized with a Tenbroek homogenizer (Corning, Corning, NY, USA) and the cell suspension was then passed through a 70µm filter. This single cell suspension was then applied to a 70/50/35% Percoll Plus (GE Healthcare, Pittsburgh, PA, USA) density gradient and spun at 2,000 g for 20-30 min. Microglia and macrophages collected at the interphase were then washed with PBS before proceeding.

**Flow Cytometry**

Isolated immune cells were stained with Live/Dead Fixable Blue Dead Cell Stain Kit from Life Technologies (Grand Island, NY, USA) to exclude dead cells. Anti-mouse CD16/CD32 mAb from BD Biosciences (San Jose, CA, USA) was used to block nonspecific Fc receptor binding and incubated for 20min at 4°C. Cells were immunostained for 30min at 4°C with surface antibodies (*Table 4.1*), fixed and permeabilized with BD Biosciences Cytofix Cytoperm Plus according to manufacturer’s protocol and stained for intracellular CD68 for 30min at 4°C (*Table 4.1*). Data were
acquired on a BD Biosciences LSR II instrument and analyzed using FlowJo v10.1 software (Ashland, OR, USA).

**Tissue Preparation and Confocal Microscopy**

Mice were anesthetized and cardiac perfused with PBS/heparin to clear blood cells from the vasculature. Brains were dissected out, meninges removed, fixed in 4% paraformaldehyde (Boston Bioproducts, Ashland, MA, USA) for 3-4h at room temperature then cryopreserved in 30% sucrose overnight at 4°C. Tissue was then placed in OCT Compound (Tissue Tek, Torrance, CA, USA) and frozen at -20°C. 12-14μm sagittal sections were cut using a Leica LM3050S cryostat (Buffalo Grove, IL, USA). Tissue sections were washed with PBS, blocked in 1% BSA (Fisher BioReagents, Fair Lawn, NJ, USA) and 1% normal goat serum (Invitrogen, Carlsbad, CA, USA) with 0.3% Triton X-100 (Sigma) at room temperature for 2h, stained overnight with the appropriate primary antibodies (**Table 4.1**) at 4°C, washed, stained with appropriate secondary antibodies (**Table 4.1**) for 1 hour at room temperature and mounted with Prolong Gold Antifade Reagent (Invitrogen). Images were acquired using either an LSM 700 scanning confocal microscope with Zeiss Imager.Z2 or a Zeiss Observer.Z1 confocal microscope equipped with Zen Blue acquisition software (Zeiss, Oberkochen, Germany).
Table 4.1 Flow cytometry and immunofluorescence antibodies.

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Secondary antibodies from Life Technologies.

Analysis of Microglia Morphology

Microglia soma area and the perimeter length of microglial extensions were measured using Fiji v1.0. Soma were measured by drawing a circumferential outline along the edge of the cell body to the base of any cellular extensions. Perimeter length was measured by summing the lengths of all cellular extensions.
RNA Extraction and qPCR

Brain tissue was dissected from mice perfused with PBS/heparin and the hippocampus, cerebellum and cortex tissue were stored in RNA Later. The cortex tissue was defined as the superficial tissue (from the surface to approximately 0.5mm deep) dissected approximately 2mm anterior, 2mm posterior and 3mm lateral with respect to bregma, a region that encompasses much of the motor and somatosensory cortex. RNA was extracted using the miRNeasy Kit (Qiagen, Germantown, MD, USA) with on-column DNase digestion (Zymo Research, Irvine, CA). Concentration was determined using a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA) and 1µg RNA was used for cDNA reverse transcription (BioRad, Hercules, CA, USA). Quantitative real-time polymerase chain reaction (qPCR) was completed using SYBR Green polymerase (BioRad) and expression measured on a BioRad CFX96 Real Time System. qPCR primers are listed in Table 4.2 and expression was quantified by using the $2^{-\Delta\Delta C_T}$ method.
### Table 4.2 Real-time PCR primers.

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### Proinflammatory

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### CCR2/5 Network

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Nanostring Gene Expression Analysis

RNA was extracted as above. RNA and Nanostring reactions were prepared according to the manufacturer’s recommendation for the Mouse nCounter Inflammation Panel (Nanostring Technologies, Seattle, WA, USA). Data were analyzed using nSolver Analysis Software 3.0 (Nanostring Technologies).

Statistical Analysis

Statistical significance was determined using student t-test or ordinary one-way ANOVA with Tukey’s multiple comparison post-test to compare the means of multiple groups. Data are shown as mean +/- SEM and were considered statistically significant at $p<0.05$. GraphPad Prism 7.0c (GraphPad Software Inc., La Jolla, CA, USA) was used for analysis.
Results

Alcohol induces recruitment of peripheral macrophages into the CNS

In order to investigate the role of peripheral infiltrating macrophages (IMs) in chronic alcohol, we used a common model of chronic alcohol consumption in mice (Fig 4.1A). We measured mRNA expression of the chemokine receptors Ccr2 and Ccr5 and the macrophage chemokine Ccl2, a ligand for CCR2, in the CNS. Alcohol induced Ccl2, Ccr2 and Ccr5 expression in both the cerebellum and hippocampus, but not in the cortex (Fig 4.1B). To test if this upregulation of monocyte chemokines and receptors is associated with infiltration of macrophages, we next isolated CNS immune cells from the total brain and examined the cells using flow cytometry. Peripheral IMs, defined by flow cytometry as CD11b⁺CD45hi cells, and differentiated from CD11b⁺CD45lo microglia, were about twice as abundant in alcohol-fed compared to pair-fed mice (5.5 ± 2.1% versus 3.24 ± 0.7%, p = 0.02) (Fig 4.1C). These data suggested that peripheral immune cells indeed infiltrate into the CNS after alcohol feeding and, based on the differential expression of chemokine receptor and ligand, that they may be recruited to particular brain regions.

To better understand the regional distribution of peripheral macrophages identified using flow cytometry from the total brain, we crossed CX3CR1<eGFP/eGFP and CCR2<RFPRFP/RFP mice to yield CX3CR1<eGFP/+ CCR2<RFPRFP/+ mice (Fig 4.1D), allowing for the differentiation of microglia (CX3CR1<eGFP>) and IMs (CCR2<RFPRFP>). CCR2<RFPRFP> macrophages were identifiable in the cortex, cerebellum and the hippocampus (Fig 4.1E). Although there was a general trend toward an increase in the number of peripheral CCR2<RFPRFP> IMs.
after chronic alcohol in all three brain regions, IMs were approximately twice as numerous in the hippocampus compared to pair-fed mice (Fig 4.1F). Interestingly, there was no change in the number of CX3CR1<sup>eGFP<sup>+/+</sup></sup> microglia in any of the brain regions (Fig 4.1G).
A) Euthanize 09:00 - 12:00

Day: -5 0 7 14 21 28 35 42

Ramp up 5% 5% EtOH

B) Chemokine/Receptor Expression

- Cortex
- Cerebellum
- Hippocampus

Relative expression (Gapdh)

C) Pair-fed

EtOH

CD45

CD11b

Infiltrating Macrophages (CD11b+ CD45+)

p < 0.01

% Live Cells

D) CX3CR1<sup>GFP</sup>/CX3CR1<sup>+/+</sup>

CCR2<sup>RFP/RFP</sup>

E) Cortex, Cerebellum, Hippocampus

F) Infiltrating Macrophages CCR2<sup>RFP</sup> Cells

RFP Cell/Area

0.00 0.05 0.10 0.15 0.20

Cortex Cerebellum Hippocampus

0.07 0.12 0.12

* G0.05

G) Microglia CX3CR1-GFP Cells

GFP<sup>*</sup> Cell/Area

0.00 0.50 1.00 1.50 2.00 2.50

Cortex Cerebellum Hippocampus

0.10 n.s. n.s.

* G0.05
Figure 4.1 Alcohol induces infiltration of peripheral macrophages into the CNS.
A) Brain immune cells from wild-type mice fed chronic alcohol (EtOH) or a pair-fed (PF) control diet were isolated and quantified by flow cytometry gating for live, single cells. Microglia (CD11b+CD45lo) were differentiated from peripheral macrophages (CD11b+CD45hi) based on surface marker staining. B) Quantification of infiltrating macrophages in pair- versus alcohol-fed mice. C-F) CX3CR1<sup>eGFP</sup>+ CCR2<sup>RFP</sup>+ mice were generated by crossing CX3CR1<sup>eGFP/eGFP</sup> CCR2<sup>+/+</sup> and CX3CR1<sup>+/+</sup> CCR2<sup>RFP/RFP</sup> mice to allow visualization of resident microglia (GFP<sup>+</sup>; green) and brain infiltrating macrophages (RFP<sup>+</sup>; red) in the cortex (D), cerebellum (E) and hippocampus (F) of mice fed chronic alcohol or a calorie-matched diet. Acquired 10X images were stitched together to provide the larger images shown. G-H) Quantification of resident GFP<sup>+</sup> microglia and RFP<sup>+</sup> brain infiltrating macrophages in the cortex, cerebellum and hippocampus. I) mRNA expression of Ccr2 and Ccr5 was measured from the hippocampus of PF and EtOH mice. Data are mean +/- SEM, n=4-7 mice/group. *p < 0.05; n.s., not significant by student’s t-test.
Inhibition of CCR2/5 signaling with Cenicriviroc (CVC) reduces CNS macrophage infiltration

In order to investigate the role of IMs that infiltrate into the CNS after chronic alcohol, we used a small molecule inhibitor, Cenicriviroc (CVC), that blocks the chemokine receptors CCR2 and CCR5, receptors expressed on monocytes and macrophages, among other immune cells. Some mice were fed chronic alcohol and were treated daily with CVC throughout alcohol exposure, while other mice fed chronic alcohol were only treated with CVC for the final three weeks, mimicking a clinical treatment paradigm where treatment often begins only after exposure has occurred. In both cases, CVC blocked alcohol-induced infiltration of CD11b+CD45^hi IMs, reducing the number to the level of pair-fed mice. Pair-fed and alcohol-fed mice received daily vehicle control injections.

Treatment with CVC alters expression of activation markers in microglia but not in infiltrating macrophages

Previously, we and others have shown that proinflammatory cytokine expression is increased in the brain after chronic alcohol exposure. Because of the proinflammatory environment in the CNS induced by alcohol consumption, we hypothesized that microglia and macrophages may have altered expression of surface activation markers. Using flow cytometry of isolated CNS immune cells from the total brain, we observed that alcohol modestly altered expression of multiple investigated activation markers, including CD86, CD68, MHC-II, CD163 and CD206.
CD68 expression was downregulated in microglia following alcohol consumption, while the remainder of the activation markers were not significantly altered, although both CD86 and CD163 showed a trend toward increased expression in alcohol-fed mice. However, CVC treatment increased both CD86 and CD68 in both treatment paradigms and reduced expression of MHC-II and CD163 (Fig 4.2D). Although CD11b^+CD45^{hi} macrophages were increased in number by alcohol treatment and this increase was abrogated by CVC administration (Fig 4.2C), some CD11b^+CD45^{hi} were detected in all groups and we therefore measured expression of activation markers in these cells. Expression of these markers was not significantly changed on CNS IMs, although an increasing trend was observed for CD86 and a decreasing trend for CD163, both of which were trends also observed in microglia. CVC treatment did not significantly alter expression of these markers on CD11b^+CD45^{hi} macrophages (Fig 4.2E).
Figure 4.2 Inhibition of CCR2/5 signaling reduces CNS macrophage infiltration and alters expression of activation markers in resident microglia but not infiltrating macrophages. A) Mice received a pair-fed diet (PF) or chronic alcohol (EtOH) and some alcohol-fed mice received six weeks of daily preventive subcutaneous CVC injection (EtOH + 6wk CVC) or three weeks of daily CVC treatment (EtOH + 3wk CVC). PF and EtOH mice received daily vehicle control injections B) Representative flow cytometry plots of microglia (CD11b+ CD45lo) and peripheral macrophages (CD11b+ CD45hi). C) Quantification of infiltrating brain macrophages (CD11b+ CD45hi) in pair-fed, alcohol-fed and treatment groups. D-E) Peripheral macrophage and resident microglia expression of various activation markers were measured by flow cytometry (CD86, CD68, MHC-II, CD163 and CD206). Data are mean +/- SEM, n=6-7 mice/group. *p < 0.05 by one-way ANOVA.
Alcohol induces morphologic changes in microglia that are partially restored by CCR2/5 inhibition

Activated microglia often assume an amoeboid morphology that is characterized by shortened cell processes and an enlarged soma. We used immunofluorescent staining of the microglial marker IBA1 to investigate if chronic alcohol induces morphological changes in line with an activated cell shape. We focused on hippocampal microglia specifically because the significant infiltration of peripheral CCR2\textsuperscript{RFP} macrophages to this region (Fig 4.1F) suggested this as a site of active neuroinflammation. We found that chronic alcohol tended to increase the cell soma size of microglia (Fig 4.3B). Inhibiting CCR2/5 signaling with CVC significantly reduced the soma in chronic alcohol-fed mice with three weeks of CVC treatment. Chronic alcohol significantly reduced the process length of hippocampal microglia and CVC administration did not affect this morphology compared to chronic alcohol-fed mice (Fig 4.3C). These data indicate that chronic alcohol induces a more amoeboid morphology than in pair-fed control mice and CCR2/5 inhibition modestly restores a normal cell body.
Figure 4.3 Alcohol-induced morphologic changes in microglia are partially restored by CCR2/5 inhibition. A) Representative images of hippocampal microglia (GFP+; green) displaying altered morphologies in pair-fed (A) versus alcohol-fed (B) mice. B) Quantification of average hippocampal microglia soma area (pixel²) and C) perimeter distance (pixel length) in mice fed control or alcohol diet or alcohol-fed mice treated with CCR2/5 inhibitor. Data are mean +/- SEM, n=3 mice/group and 3-10 sections/mouse. *p < 0.05 by one-way ANOVA.
Chronic alcohol induces inflammatory protein and gene expression changes

We measured protein levels of proinflammatory cytokines including TNFα, IL-1β and IL-6 in the hippocampus, where we had observed a significant increase in CCR2+ macrophages following chronic alcohol. We found that both TNFα and IL-1β (including both cleaved and uncleaved forms) were induced by chronic alcohol and that three-week treatment with CVC reduced these proteins as well as IL-6 in CVC-treated alcohol-fed mice (Fig 4.4A).

To further probe the expression of proinflammatory cytokines and related immune genes, we used the Nanostring Inflammation Panel to screen mRNA levels of 254 inflammation-related genes. In this screen, we found 17 immune-related genes that were significantly altered in the cerebellum (Fig 4.4B). We followed this screen with qPCR quantification of these gene targets as well as other selected proinflammatory targets and chemokine ligands and receptors associated with CCR2/5 signaling (Fig 4.4C). Including more samples as well as samples from mice treated with CVC, we found that gene expression was significantly altered in the hippocampus, cortex and cerebellum and that CVC treatment corrected some of these alterations (Fig 4.4C). Prominent upregulation in the CCR2/5 signaling pathway were observed in alcohol-fed mice in the hippocampus where we also observed the most infiltration of CCR2+ peripheral macrophages. Table 4.3 provides the mean and standard deviation for each gene and group in the cortex, hippocampus and cerebellum as well as p-value comparisons between pair- and alcohol-fed mice (PF v EtOH) as well as alcohol-fed and CVC-treated alcohol-fed mice (EtOH v
6wk CVC; EtOH v 3wk CVC). Significant differences are denoted with an * and are offset in the column (Table 4.3).
Figure 4.4 Chronic alcohol induces inflammatory gene expression changes in multiple brain regions. A) Proinflammatory cytokines TNF, IL-1 and IL-6 protein was measured from the hippocampus of pair- (PF) and alcohol-fed (EtOH) as well as alcohol-fed mice treated with CVC for three weeks (3wk) by ELISA. B) Inflammatory gene expression of pair- and alcohol-fed mice was analyzed using Nanostring nCounter Immunology Panel and revealed 17 genes significantly altered in the cerebellum (green, expression increased; red, expression decreased). C) Gene expression changes of genes found by Nanostring to be altered as well as some common inflammatory markers and CCR2/5 axis genes were measured in the hippocampus, cortex and cerebellum by qPCR in a larger cohort of pair- and alcohol-fed mice as well as mice treated with 3 or 6 weeks CVC (purple, >3-fold increase in expression; cyan >3-fold decrease in expression compared to PF; complete gene expression profile data found in Table 4.3). Data are mean +/- SEM, A) n=4-6 mice/group; B) n=3 mice/group; C) n=5-6 mice/group. *p < 0.05 by one-way ANOVA.
Table 4.3 Gene expression profile of the cortex, hippocampus and cerebellum.

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Table 4.3 (cont.) Gene expression profile of the cortex, hippocampus and cerebellum.

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Gene expression was measured by qPCR. Data are mean or standard deviation (Stdev), n=5-6 mice/group. *p < 0.05 and significant p-values are offset from non-significant values.
148
Table 4.3 (cont.) Gene expression profile of the cortex, hippocampus and
cerebellum.

Gene expression was measured by qPCR. Data are mean or standard deviation (Stdev),
n=5-6 mice/group. *p < 0.05 and significant p-values are offset from non-significant
values.


Alcohol increases excitatory VGLUT1 and HOMER1 synapses in the hippocampus

Having observed significant macrophage infiltration and proinflammatory cytokine expression in the hippocampus, we next asked if these immune-related changes may also be associated with changes in neural connectivity and synapses. First we looked at excitatory synapses by staining for the presynaptic glutamate transporter VGLUT1 and the postsynaptic density protein HOMER1 (Fig 4.5A). Chronic alcohol significantly upregulated both VGLUT1 (Fig 4.5B) and HOMER1 (Fig 4.5C) in the hippocampus (All HPC) as well as in various subregions of the hippocampus including the CA1, CA3 and the dentate gyrus (DG). We provide aggregated HPC data to allow for comparison with prior studies that did not differentiate hippocampal subregions. Because these proteins are expected to be expressed in close proximity when forming an excitatory synapse, we measured the colocalization of VGLUT1 and HOMER1 and found that alcohol also increased the total area of overlap (Fig 4.5D), indicating an increase in excitatory synapses.

At the transcriptional level, chronic alcohol also induced the expression of Slc17a7, the gene encoding VGLUT1 (Fig 4.5F). Interestingly, CVC treatment for three weeks further induced Slc17a7 expression. Alcohol did not increase the expression of Slc7a6 which encodes VGLUT2, another excitatory presynaptic protein (Fig 4.5F). Similar to the histologic staining, chronic alcohol induced expression of Homer1 compared to pair-fed animals (Fig 4.5G). CVC treatment did not change the expression of Homer1 with either treatment approach (Fig 4.5G).
Figure 4.5 Alcohol increases excitatory VGLUT1 and HOMER1 synapses in the hippocampus. A) Representative images of hippocampal sections stained for VGLUT1 (green) and HOMER1 (magenta) from pair-fed (PF) and alcohol-fed (EtOH) mice. B) Quantification of relative VGLUT1 positive staining area in the hippocampal (HIPP) CA1, CA3 and DG regions and throughout the HIPP. C) Quantification of HOMER1 in various hippocampal regions. D) VGLUT1 and HOMER1 colocalization in the hippocampus. E-G) Relative mRNA expression of Slc17a7 and Slc17a6 (encoding VGLUT1 and VGLUT2 proteins) as well as Homer1 in the hippocampus. Data are mean +/- SEM, n=3-4 mice/group with 4-5 images per region per mouse in A-D); n=6-7 mice/group in E-G). *p < 0.05; n.s., not significant by student’s t-test or by one-way ANOVA.
Inhibitory VGAT and Gephyrin synapses in the hippocampus are increased after chronic alcohol

Next, we investigated whether chronic alcohol influences inhibitory hippocampal synapses by staining for the presynaptic GABA transporter VGAT and the postsynaptic structural protein Gephyrin (Fig 4.6A). Chronic alcohol did not significantly alter the expression of VGAT in CA1 or CA3 although expression in the DG was significantly reduced after alcohol consumption (Fig 4.6B). Interestingly, Gephyrin expression was increased in CA1 and CA3 but not the DG (Fig 4.6C). VGAT and Gephyrin colocalization was unchanged in CA1, significantly increased in CA3 and trended toward decreased in DG (Fig 6D). Vgat mRNA expression was increased from the total hippocampus after chronic alcohol feeding, with no effect in mice treated with CVC (Fig 4.6E). Gephyrin expression was not significantly changed by chronic alcohol, although CVC six-week treatment did decrease expression (Fig 4.6F). Taken together, these data suggest that unlike the robust changes induced by alcohol in excitatory synapse proteins, chronic alcohol has modest impact on inhibitory synapses in the hippocampus.
Figure 4.6 Alcohol increases inhibitory VGAT and Gephyrin synapses in the hippocampus. A) Representative images of hippocampal sections stained for VGAT (green) and Gephyrin (magenta) from pair-fed (PF) and alcohol-fed (EtOH) mice. B) Quantification of relative VGAT positive staining area in the hippocampal (HIPP) CA1, CA3 and DG regions and throughout the HIPP. C) Quantification of Gephyrin in various hippocampal regions. D) VGAT and Gephyrin colocalization in the hippocampus. E-F) Relative hippocampal mRNA expression of Vgat and Gephyrin. Data are mean +/- SEM, n=3-4 mice/group with 4-5 images per region per mouse in A-D); n=6-7 mice/group in E-G). *p < 0.05; n.s., not significant by student’s t-test or by one-way ANOVA.
Complement component C1q colocalizes with VGLUT1 in the hippocampus of mice fed chronic alcohol

Complement signaling is classically described as an inflammatory cascade, but it has also been identified as an important component of synapse development and in particular pruning\textsuperscript{158,159}. Because we observed macrophage infiltration, inflammatory cytokine and chemokine expression and upregulation of excitatory synapses in the hippocampus, we investigated the expression of the complement component C1q along with the excitatory synapse marker VGLUT1 (Fig 4.7A). C1q expression was increased in the DG (Fig 4.7B). Colocalization of C1q and VGLUT1 was significantly increased in the DG (Fig 4.7C). We also measured $C1qa$ and $C1qb$, which encode two components of the C1q protein (Fig 4.7D). $C1qa$ expression in the hippocampus was unchanged by chronic alcohol while $C1qb$ was upregulated. Treatment with six-week CVC reduced $C1qa$ expression and had no impact on $C1qb$ expression.
Figure 4.7 Complement component C1q colocalizes with VGLUT1 in the hippocampus of mice fed chronic alcohol. A) Representative images of hippocampal sections stained for VGLUT1 (green) and C1q (magenta) from pair-fed (PF) and alcohol-fed (EtOH) mice. B) Quantification of relative C1q positive staining area in the hippocampal (HIPP) CA1, CA3 and DG regions and throughout the HIPP. C) VGLUT1 and C1q colocalization in the hippocampal CA1, CA3 and DG regions and throughout the HIPP. D) Relative hippocampal mRNA expression of C1qa and C1qb. Data are mean +/- SEM, n=3-4 mice/group with 4-5 images per region per mouse in A-D); n=6-7 mice/group in E-G). *p < 0.05; n.s., not significant by student’s t-test or by one-way ANOVA.
Complement protein C3 is increased in the hippocampus following chronic alcohol consumption and colocalizes with VGLUT1

Another complement protein, C3, has also been shown to play an important role in synapse development and pruning\textsuperscript{157,159}. Again, we stained for C3 and VGLUT1 (Fig. 4.8A) and observed significantly increased expression of C3 in CA1, CA3 and DG of the hippocampus (Fig. 4.8B). C3 colocalization with VGLUT1 was also significantly increased in CA1, CA3 and DG (Fig. 4.8C). This pronounced increase in C3 protein, coupled with the modest increase in C1q expression, coincides with an increase in many other neuroinflammatory genes and proteins, including pro-inflammatory cytokines. Interestingly, the colocalization of complement proteins and the synaptic protein VGLUT1 also suggests that complement is marking some synapses for pruning and, since both excitatory and inhibitory synapses were increased after chronic alcohol, that this pruning process may be disrupted.
Figure 4.8 Alcohol upregulates complement C3 that colocalizes with VGLUT1 in the hippocampus. A) Representative images of hippocampal sections stained for VGLUT1 (green) and C3 (magenta) from pair-fed (PF) and alcohol-fed (EtOH) mice. B) Quantification of relative C3 positive staining area in the hippocampal (HIPP) CA1, CA3 and DG regions and throughout the HIPP. C) VGLUT1 and C3 colocalization in the hippocampal CA1, CA3 and DG regions and throughout the HIPP. Data are mean +/- SEM, n=3-4 mice/group with 4-5 images per region per mouse in A-D); n=6-7 mice/group in E-G). *p < 0.05 by student’s t-test.
**CD68 expression is downregulated by chronic alcohol**

CD68 is a lysosomal protein marker that is often used in association with measurements of phagocytosis in macrophages and particularly microglia\(^{157,320}\). We hypothesized that because both excitatory and inhibitory synapses were increased in density in the hippocampus, perhaps microglial levels of CD68 may also be altered. Microglial CD68 expression, measured by immunofluorescence was decreased in the hippocampus of alcohol-fed compared to pair-fed mice (Fig 4.9A-B). This observation was corroborated by flow cytometric analysis of isolated microglia in which we also observed a decrease in microglial CD68 positivity in alcohol-fed mice (Fig 4.9C). Interestingly, treatment with CVC did not affect microglial CD68 expression (measured as colocalization of CD68 and the microglial marker IBA1) except in the CA3 region of the hippocampus where CVC decreased CD68 expression (Fig 4.9B). However, flow cytometry measurements of CD68\(^{+}\) microglia revealed an effect of CVC treatment as both inhibitor treatment paradigms increased CD68 positivity (Fig 4.9C). Importantly, the flow cytometry is based on microglia from the total brain, whereas the immunofluorescence measurements were isolated only in the hippocampus. Taken together, these data suggest that alcohol significantly reduces microglial CD68 expression and provides evidence of microglial alterations that may impact synapse density in the hippocampus after chronic alcohol exposure.
Figure 4.9 Alcohol downregulates microglial expression of CD68. A) Representative images of hippocampal sections stained for Iba1 (green), CD68 (red) and DAPI (blue) from pair-fed (PF), alcohol-fed (EtOH) and CVC-treated alcohol-fed mice. B) Quantification of Iba1 and CD68 colocalization, normalized to total Iba1+ area per image in the hippocampal CA1, CA3 and DG regions and throughout the HPF. C) Flow cytometry data showing the percent of microglia (defined as CD11b+ CD45lo) that are positive for CD68 expression. Data are mean +/- SEM, n=3-4 mice/group with 4-5 images per region per mouse in A-D); n=6-7 mice/group in E-G). *p < 0.05; n.s., not significant by one-way ANOVA.
Discussion

Previous research has described neuroinflammation associated with chronic alcohol consumption. We hypothesized that alcohol could induce macrophage infiltration into the brain and that this infiltration could drive the neuroinflammation observed after chronic alcohol. We therefore tested if blockade using a CCR2/5 dual inhibitor of the chemokine network associated with macrophage chemoattraction could reduce alcohol-induced neuroinflammation. Here, we show that chronic alcohol induces region-specific infiltration of IMs into the CNS that is associated with cytokine expression and microglial activation. Inhibition of CCR2/5 signaling using the small molecule inhibitor CVC abrogated the infiltration of macrophages, reduced cytokine expression and partially normalized microglial morphology. We also provide evidence of an increase in the number of excitatory and inhibitory synapses in the hippocampus following chronic alcohol administration.

Although the CNS was long considered an immune privileged compartment, an appreciation for peripheral immune infiltration in the setting of disease has been accepted. Only recently has infiltration of peripheral macrophages been described in the setting of alcohol-induced neuroinflammation. Using a different alcohol model than previously studied in mice, we confirm this observation using flow cytometry of total immune cells in the brain, detecting an increase in CD11b+CD45hi macrophages. Peripheral macrophages have previously been distinguished from microglia (CD11b+CD45lo) and although expression of CD45 can shift during inflammation, gene expression studies suggest its expression level remains a distinguishing feature between the two cell types. Additionally, we created CX3CR1eGFP/+ CCR2RFP/+ mice to allow
for visualization and localization of infiltrating CCR2+ macrophages. While we observed a trend toward increased macrophages in the cortex and cerebellum, the largest alcohol-induced increase observed was in the hippocampus, a region of significant alcohol-related inflammation in both rodents and humans\textsuperscript{142,184,185}. These data suggest that alcohol-induced macrophage infiltration into the CNS may be region-specific and possibly linked to localized neural damage and immune signaling.

Blockade of CCR2/5 signaling with CVC successfully limited the alcohol-induced infiltration of peripheral macrophages into the CNS. Previously, CVC was shown to be effective at limiting macrophage chemotaxis to the liver in a model of fibrosis\textsuperscript{225}. While successful inhibition of peripheral immune cell infiltration is consistent with blockade of a chemokine receptor, blocking CCL2 signaling has additional advantages. In the CNS of CCL2 knock out mice, production of proinflammatory cytokines TNF\textalpha and IL-1\textbeta was significantly reduced after peripheral injection of bacterial endotoxin (lipopolysaccharide; LPS)\textsuperscript{322}. Interestingly, these proinflammatory cytokines were expressed even before peripheral cell infiltration occurred, suggesting that, at least in this model, neuroinflammatory gene expression preceded the response of the peripheral immune system. This has important implications for our present study, using the small molecule CVC, which may cross the blood-brain barrier and block CCL2 signaling in CNS resident cells in addition to its ability to inhibit chemotaxis of peripheral immune cells. Reducing inflammatory signaling in the CNS, which in the case of LPS precedes immune cell infiltration, may contribute to the reduced expression of chemokines. Cell-specific knockout of CCR2 (such as using LysM-driven knockout in
peripheral macrophages or CX3CR1-driven knockout in microglia, for example) could help to elucidate the importance of peripheral blockade of CCR2 versus central signaling. An important caveat to our study is that we did not examine the effect of CVC in pair-fed animals. Although we would not expect to see significant influence in these mice, without activation of inflammatory signaling or immune cell attraction, this could be included in future studies to better understand how CCR2/5 blockade may affect baseline peripheral and organ-specific immunity.

Additionally, recent evidence suggests that, in the developing brain, mice deficient in either CCL2 or CCR2 are protected from alcohol-induced neuroinflammation (including proinflammatory cytokine expression and microglial activation) and neurotoxicity when treated with an acute alcohol exposure at postnatal day four. These results are in agreement with the present data, which importantly is from adult mice with chronic alcohol consumption, and provide further evidence for the importance of CCL2/CCR2 signaling in alcohol-induced neuroinflammation. Further experiments, including using CCL2 and CCR2 knockout adult mice under chronic alcohol conditions will also be informative.

Interestingly, we observed the most pronounced alterations in surface activation markers between microglia of alcohol-fed mice and microglia from mice treated with CVC. A possible explanation for this is that in the absence of IMs, the remaining microglia are forced to assume a more activated phenotype and respond to the damage induced by alcohol. Interestingly, while we observed surface protein activation markers in microglia, we also observed an activated morphologic phenotype with increased soma
size and reduced microglial cell process length. For this analysis, we focused on microglia in the hippocampus, the site of significant peripheral macrophage infiltration (Fig 4.1F) and an increase in protein and mRNA expression of proinflammatory cytokines (Fig 4.4). Microglial morphology is one indicator of an inflammatory milieu, as these cells change their shape in response to immune activation, and our flow cytometry data suggested that microglia are significantly affected by alcohol and CVC administration (Fig 4.2). CVC treatment partially rescued microglial morphology. Compared with alcohol-fed untreated mice, the CVC treatment groups had slightly larger cell bodies but retained the shortened cell process phenotype of alcohol-fed mice. This mixed microglial phenotype, despite the absence of IM infiltration, likely represents the innate response of microglia to alcohol which is characterized by activation and production of proinflammatory cytokines and reactive oxygen species.\(^{187,324,325}\). The imaging in Fig 4.3 suggests that microglial morphology was altered by chronic alcohol exposure although histologic examination of the complete microglial architecture is technically limited as some microglial processes may be disrupted by the histologic preparation. Therefore, we examine microglial morphologic changes in the context of other measures of a neuroinflammatory milieu including analysis of activation markers and proinflammatory gene expression.

Previous research has sought to characterize gene expression changes caused by chronic alcohol in various parts of the brain. Gene expression analyses in human\(^{243,326-328}\) and rodents\(^{329}\) reveal alterations in genes related to neurons and neurogenesis, axonal growth, myelin regulation, intracellular signaling, protein trafficking and other critical
cell processes. Interestingly, neuroimmune genes were significantly increased in the frontal cortex of human patients with alcoholism. Using a targeted screen, we observed alterations in inflammation-related transcripts in the CNS after chronic alcohol in mice and expanded our focus to investigate the hippocampus, cortex and cerebellum. We observed upregulation of multiple proinflammatory genes similar to previous descriptions and some of these were altered by the treatment of CVC to block CCR2/5 signaling. As noted previously, some cytokine expression is increased prior to the infiltration of peripheral immune cells in other neuroinflammation models, which may provide an explanation for why CVC treatment only altered expression of some inflammatory genes in the brain regions investigated.

Our data suggest that infiltrating macrophages specifically target the hippocampus as a site of increased infiltration. The hippocampus is a critical center for learning and memory and has been implicated in the pathology associated with AUD for decades. Hippocampal volume loss is associated with alcohol consumption in a dose dependent manner. In a recently published study, researchers followed individuals for over three decades and show that increasing amounts of alcohol consumption are associated with greater risk of hippocampal atrophy. Alcohol also influences key processes in memory formation and learning by suppressing long-term potentiation (LTP) of synaptic connections and altering proper maturation and maintenance of dendritic spines and synaptic connections within the hippocampus.

In this study, we observed that chronic alcohol induces expression of presynaptic excitatory protein VGLUT1, as well as postsynaptic marker for excitatory synapses
HOMER1, and that these proteins colocalize, suggesting an increase in excitatory synaptic connections in the hippocampus. This observation is partially corroborated by Montesinos et al. who recently observed induction of the excitatory postsynaptic protein PSD-95 in the prefrontal cortex in a mouse model of adolescent binge drinking\textsuperscript{195}. In their study, TLR4 KO mice did not show an alcohol-related induction of PSD-95. PSD-95, like HOMER1, is part of the postsynaptic density, a cluster of postsynaptic proteins important in structural scaffolding and likely other function at the postsynaptic terminal\textsuperscript{330,331}. Additionally, Klenowski et al. have observed increased excitatory synaptic activity as well as an increase in dendritic spine densities following binge alcohol administration in rats\textsuperscript{194}. Although the models (binge versus chronic) and brain regions (prefrontal cortex versus hippocampus) examined differ between these two studies and the data presented here, the common observation of increased excitatory synaptic proteins suggests that alcohol consumption leads to an alteration in synapse density regulation.

While a firm date of adulthood has not been agreed upon by the scientific community, mouse adulthood is generally considered to begin after sexual maturity sometime around twelve weeks of age\textsuperscript{332}. In our study, we began alcohol feeding in eight-week-old female mice (approximately post-natal day 56) and sacrificed 47-50 days later (between fourteen- and fifteen-weeks-old). Prior to adulthood, in adolescence, significant synaptic pruning occurs in various brain regions that are also implicated in alcohol addiction, including the amygdala, parts of the striatum and the prefrontal cortex\textsuperscript{333-335}. While pruning continues into adulthood and is an important part of memory formation\textsuperscript{336}, disruption of the proper developmental synaptic pruning process by the introduction of
alcohol at an age so close to the end of adolescence could account for the increased excitatory (VGLUT1/HOMER1) and inhibitory (VGAT/Gephyrin) synapses reported here. Indeed, adolescent but not adult alcohol consumption is associated with altered neuronal firing\textsuperscript{337,338} and neuron viability\textsuperscript{339-341} in the hippocampus, which could impact plasticity and synapse maturation.

Interestingly, we have observed an increase in colocalized pre- and postsynaptic proteins while at the same time there is an increase in complement protein colocalized with VGLUT1 in the hippocampus of alcohol fed mice. Current understanding of complement proteins and synapses largely draws from developmental pruning in which microglia engulf synapses that are tagged with complement protein\textsuperscript{157,158}. However, in our study in older mice (alcohol exposure for six weeks began in mice aged six- to eight-weeks old), developmental pruning is unlikely to be continuing. Additionally, we observe an increase rather than a decrease in synapses. However, mice in this study were sacrificed at one time point and it is possible that synapse numbers may decrease after further alcohol exposure or after alcohol withdrawal. Indeed, Montesinos et al. describe an increase in excitatory synapses using an adolescent binge drinking model\textsuperscript{195}. However, Risher et al. observed that mice treated under a similar adolescent binge drinking paradigm, but allowed to live into adulthood in abstinence, had reduced synapse density suggesting that there is likely a time course involved in the dysregulation of synapses following alcohol exposure\textsuperscript{138,139}.

Increased complement expression in the hippocampus not only coincided with an increase in synapse density but also with an upregulation of many proinflammatory
cytokines. Complement proteins make up an important signaling cascade that becomes activated in the setting of inflammation, making the observed increase consistent with a proinflammatory state in the CNS following alcohol exposure. Interestingly, there is evidence that proinflammatory cytokines may have influence on synapse function, much like complement involvement in the regulation of synapse development. For example, TNFα has been shown to modulate glutamate receptors and decrease synaptic strength. Interestingly, while the resident source is likely from microglia, peripheral macrophages also express TNFα and may be involved in influencing synapses in addition to their possible role promoting neuroinflammation. Further study, using either CVC-treated animals or genetic knockout (e.g., CCR2) mice in which peripheral macrophages are blocked from the CNS, will be critical to understanding the contributions of infiltrating macrophages on synaptic dysregulation (including through either cytokine release or direct involvement in synaptic engulfment similar to microglia) in alcohol-exposed animals.

Microglia play a critical role in the synaptic pruning process and alterations in their normal function have been shown to disrupt proper developmental synaptic pruning. In our study, alcohol consumption led to morphologic changes in microglia (Fig 4.3) and changes in surface marker expression (Fig 4.2). Thus, the effect of alcohol on microglia is significant and may lead to altered synaptic pruning functionality. Indeed, the lysosomal protein CD68 was downregulated in hippocampal microglia. Additionally, we noted increased cytokine protein expression in the hippocampus (Fig 4.4) and cytokines have been implicated in synapse dysregulation in hippocampus neurons in...
Alcohol consumption may be inducing microglial activation and cytokine production that adversely affects the responsiveness of microglia to synaptic pruning, thus creating an environment where complement-tagged synapses are not properly engulfed.
Conclusion

In conclusion, we report here that chronic alcohol induces region-specific infiltration of IMs into the CNS and that inhibition of CCR2/5 signaling using the small molecule inhibitor CVC abrogated the infiltration of macrophages. Chronic alcohol also induced cytokine expression and microglial activation that was partially normalized by CCR2/5 inhibition. We also provide novel evidence of an increase in the number of excitatory and inhibitory synapses in the hippocampus following chronic alcohol administration. These data provide critical insights into the role of CCR2/5 signaling, IMs and microglia in alcohol-induced neuroinflammation and offer a novel target for therapeutic treatment of alcohol use disorder.
CHAPTER V

Inhibition of the inflammasome signaling cascade reduces alcohol consumption in female mice

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Abstract

Alcohol use disorder is a significant societal and medical burden that is associated with both organ pathology and addiction. Excessive alcohol use results in neuroinflammation characterized by IL-1β increase in the brain and activation of the inflammasome, a multiprotein complex that is involved in IL-1β production. Recent studies suggest that inflammation could contribute to alcohol addiction. Here, we targeted components of the NLRP3 inflammasome cascade, which senses and responds to immunologic stimuli, to determine if NLRP3 inhibition modulates alcohol consumption. C57BL/6J male and female mice were provided a two-bottle choice of alcohol at increasing concentrations (3, 6, 9 and 12%, four days each) or water and some were treated with daily injections of an NLRP3 inhibitor (MCC950), a caspase-1 inhibitor (VX765), IL-1 receptor antagonist (IL1ra; Anakinra), or vehicle injection. In untreated animals, female mice consumed more alcohol than males. Treatment with MCC950 and Anakinra significantly reduced alcohol consumption and preference in female mice at all alcohol concentrations (3, 6, 9 and 12%), while caspase-1 inhibition with VX765 reduced consumption in females only at lower alcohol concentrations (3 and 6%). Male mice were largely unaffected by inflammasome inhibitor treatment. These findings highlight gender differences in alcohol preference and demonstrate that inhibition of different steps in inflammasome signaling can reduce alcohol consumption in females. Inhibition of NLRP3 inflammasome activation and the inflammasome-IL-1β cascade opens novel insights into the development of new therapies to address alcohol use disorder in an era of targeted and precision medicine.
Introduction

Alcohol consumption is prevalent in our society with a quarter of American adults consuming four to five drinks in one sitting, qualifying for binge drinking behavior\(^2\). In the United States, 15 million adults and more than 500,000 adolescents have alcohol use disorder, which causes the death of 88,000 individuals per year\(^2\). Alcohol use disorder (AUD) is the third leading preventable cause of death in the US, behind tobacco and poor diet/physical activity\(^2\). Current therapies to treat AUD vary in their therapeutic mechanism of action and include naltrexone (an opioid antagonist), acomporosate (a modulator of neurotransmitter signaling) and disulfuram (an inhibitor of acetaldehyde dehydrogenase)\(^19\). However, millions of patients continue to suffer with AUD and methods to reduce alcohol consumption, whether through changes in behavior or pharmacologic therapies, can have a significant impact on relieving a preventable, but often untreated, health burden.

Recent gene expression studies of AUD and the mechanisms involved in disease progression have highlighted the role of immune pathways, suggesting a new avenue of possible candidates for targeted treatment\(^24\). Studies in animal models showed that manipulation of various immune pathways could reduce alcohol consumption in mice\(^16\). The reverse has also been demonstrated where inducing systemic inflammation, via peripheral injection of the bacterial wall component, lipopolysaccharide (LPS), increased alcohol consumption in mice\(^25\). Interestingly, studies in human patients correlate alcohol craving severity with increased markers of systemic inflammation, including cytokines\(^29\).

We and others have previous identified interleukin-1\(\beta\) (IL-1\(\beta\)) as an important
cytokine that is increased in the brain following a chronic alcohol feeding model in mice\textsuperscript{170,179,180} and treatment with IL-1 receptor antagonist (IL-1ra) ameliorates both alcohol-induced neuroinflammation\textsuperscript{162} as well as hallmarks of alcoholic liver disease\textsuperscript{112}. IL-1β production is a result of activation of the intracellular NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome complex\textsuperscript{175}. The NLRP3 inflammasome is a multiprotein complex including NLRP3 as the sensor, apoptosis-associated speck-like protein (ASC) as the adaptor and caspase-1 (Casp1) as the enzymatic effector. Upon inflammasome activation, Casp1 becomes active and cleaves pro-IL-1β into its active form, IL-1β, which can signal to neighboring cells and beyond\textsuperscript{177,178}. Preclinical studies have revealed signaling through the NLRP3 inflammasome as a key component in alcohol-induced liver pathology in chronic alcohol mouse models\textsuperscript{70,111}. We have also shown that mice lacking functional ASC, Casp1 and IL-1 receptor are protected from alcoholic liver disease\textsuperscript{112,113}.

Based on evidence that immune signaling can impact alcohol consumption as well as the importance of NLRP3 inflammasome in alcohol-induced pathology, we hypothesized that pharmacological targeting of the inflammasome could be an effective approach to modulate AUD. Multiple agents exist for targeting of NLRP3 inflammasome components\textsuperscript{347}. MCC950 is a small molecule inhibitor that specifically targets and inhibits the inflammasome sensor NLRP3\textsuperscript{348}. VX765 inhibits the effector protein of the inflammasome, Casp1, blocking its ability to enzymatically activate its cleavage targets, including pro-IL-1β and IL-18\textsuperscript{349,350}. Anakinra is a recombinant IL-1 receptor antagonist (IL-1ra) capable of binding the IL-1 receptor (IL-1r) without inducing downstream
signaling. Thus, IL-1ra acts as a competitive inhibitor of endogenous IL-1r, thereby blocking the ability of IL-1β and/or IL-1r to carry out inflammation signaling and communication\textsuperscript{351,352.}

In this study, we aimed to test the novel hypothesis that inflammasome signaling is an important driver of alcohol consumption. Using the two-bottle choice paradigm with male and female mice, we describe the effects of three inflammasome cascade inhibitors (MCC950, VX765 and Anakinra) on alcohol consumption. These data will provide critical insight not only into the role of the inflammasome and inflammation in the behavior associated with alcohol consumption but may also offer rationale for the use of inflammasome inhibitors as adjuvants in clinical alcohol cessation therapy.
Methods

Mice

All animals were cared for in strict accordance with the approved Institutional Animal Care and Use Committee protocol specific to the procedures described in this study at the University of Massachusetts Medical School (Protocol #A-1154-14; G.S.). Wild-type C57BL/6J 6- to 8-weeks-old male and female mice were purchased from Jackson Laboratories and were cohoused in the University of Massachusetts Medical School Animal Medicine Facility for one week prior to the start of the experiment at which time they were singly housed. Mice were maintained on a 12h light/dark cycle with lights on at 7:00 AM. Inhibitor injections and feeder changes occurred each day between 1:00 and 3:00 PM.

Two-bottle choice test

A two-bottle choice between alcohol or drinking water was used to measure alcohol consumption as previously described15. Briefly, we constructed glass graduated feeder tubes as described353. Feeders were placed in the cages 2.5cm from the left wall of the cage or 1cm from the right pellet food divider with 5cm between the two bottle feeders. Chow diet was provided *ad libitum* throughout the experiment. Singly housed mice received one-day acclimation to the new feeders containing water only. Thereafter, one feeder contained alcohol and the other normal drinking water. Mice consecutively received four days each of 3, 6, 9 and 12% alcohol in drinking water. Alcohol was made fresh daily to avoid concentration fluctuations due to evaporation. The side of the alcohol
and water feeders was altered each day to control for any side preference bias. Alcohol and water volume consumed was measured daily and a control cage without mice was included on each mouse rack to account for spillage of liquid throughout the 24-hour period. Alcohol consumption was calculated by converting the volume consumed (corrected for the spilled volume from the control cage) to g of alcohol and normalizing to the kg body weight of each mouse. Preference for alcohol was calculated by dividing the volume of alcohol consumed by the total liquid intake for that 24-hour period (water plus alcohol volume consumed).

**Non-Alcohol Tastants**

After receiving 12% alcohol, alcohol exposure and inhibitor injections were ceased and mice were allowed to rest for four weeks. Following this rest phase, all inhibitors were restarted and some mice received saccharin (0.033% and 0.066% w/v consecutively for four days at each concentration; Sigma, St. Louis, MO) while others received quinine (0.03 mM and 0.06 mM consecutively for four days at each concentration; Sigma, St. Louis, MO). The side of the tastant and water feeders was changed each day to control for a side preference bias.

**Inflammasome Inhibitor Administration**

Inhibitors of various inflammasome-related molecules were resuspended, aliquoted and frozen until use. MCC950 (Cayman Chemicals, Ann Arbor, MI) was resuspended with DMSO to a stock concentration of 50 mg/mL and diluted to a working
concentration of 0.25 mg/mL in 0.9% saline. VX765 (ApexBio, Houston, TX) was resuspended with DMSO to a stock concentration of 100 mg/mL and diluted in saline to a working concentration of 1 mg/mL in 0.9% saline. Anakinra (Kineret™) was purchased in solution at a concentration of 100 mg/0.67 mL and diluted to a working concentration of 2.5 mg/mL. Inhibitors were given by intraperitoneal injection of the following doses: MCC950 5mg/kg body weight; VX765 20mg/kg body weight; Anakinra 25mg/kg body weight. 1% DMSO in 0.9% saline was used as a vehicle control injection. Some mice did not receive any injection.

**Chronic Alcohol Feeding**

All mice were fed Lieber-DeCarli (BioServ, Flemington, NJ) pair-fed diet during the first five days to become acclimated to the liquid diet. Some mice then received Lieber-DeCarli alcohol diet with maltose dextrin and increasing concentrations of alcohol from 1 to 5% over five days. Alcohol-fed mice were then maintained on 5% alcohol for four weeks. Pair-fed mice received a calorie-matched pair fed diet for the feeding duration.

**Liver Enzyme Assays**

Serum was separated from whole blood and frozen -80°C until the assay was completed. Serum alanine and aspartate transaminases (Teco Diagnostics, Anaheim, CA) were measured using a kinetic method as previously described72.
Blood Ethanol Concentration Measurements

The amount of alcohol in the serum samples was measured using an Analox Alcohol Analyzer.

Histology

Liver sections were formalin fixed, paraffin embedded and stained with hematoxylin and eosin at the UMass Medical School DERC histology core.

Statistical Analysis

Statistical analysis was carried out using GraphPad Prism Version 7.0 using Student t-test. $p < 0.05$ was considered statistically significant.
Results

Female mice consume more *ad libitum* alcohol than male mice

In a preliminary study, we tested the alcohol consumption differences between female and male C57BL/6J mice. Mice were singly housed and provided two bottles in the cage containing either plain drinking water or alcohol at increasing concentrations (3, 6, 9, 12%) in four-day intervals. Female mice consumed more alcohol than aged-matched male mice at each dose of alcohol ranging from 3 to 12% (Figure 5.1A). These results indicate significant gender differences in alcohol consumption in C57BL/6J mice. There was no statistical difference between females and males in the two-bottle choice (2-BC) test in serum alanine transaminases (ALT; 11.8 ± 4.3 vs. 14.7 ± 4.3 IU/mL, p = 0.27), aspartate transaminase (AST; 23.9 ± 5.9 vs. 23.1 ± 3.5 IU/mL, p = 0.80) or blood ethanol concentration (BEC; 2.35 ± 0.64 vs. 2.95 ± 1.9 mg/dL, p = 0.48).

Two-bottle choice test of alcohol consumption does not induce obvious liver pathology

There are multiple mouse alcohol feeding paradigms in laboratory use, including the chronic 5% alcohol feeding using the Lieber-DeCarli liquid diet for different lengths from ten days to four or more weeks. These chronic alcohol-feeding models induce elevated liver transaminases, hepatic steatosis and inflammation and often female C57BL/6J mice are used in studies for liver disease. Because we did not observe any differences in ALT, AST or BEC between female and male mice, we present data in Figure 5.1B-D only for female mice in the two-bottle choice test and chronic feeding
model. A comparison between mice fed chronic 5% alcohol in the Lieber-DeCarli liquid diet and mice exposed to the two-bottle choice paradigm revealed that mice in the two-bottle choice test had lower blood alcohol concentration than those in a chronic feeding (Figure 5.1B). Chronic 5% alcohol in liquid diet significantly elevated serum ALT and AST compared with two-bottle choice exposed mice (Figure 5.1C). H&E staining of livers from the two-bottle choice treated mice did not reveal evidence of inflammation or lipid accumulation as observed in livers from the chronic feeding paradigm (Figure 5.1D). Although the lack of profound pathology may not be surprising in the two-bottle choice paradigm, it is useful to recognize the differences between this behavioral model and the chronic 5% Lieber-DeCarli paradigm.
Figure 5.1 Female mice drink more alcohol than males but 2-BC induces less liver damage and steatosis than chronic alcohol exposure. A) In a preliminary study, male and female mice were singly housed and provided *ad libitum* water or alcohol (EtOH) in drinking water at various concentrations of 3%, 6%, 9% and 12% for four days each (two-bottle choice test, 2-BC). Consumption was measured daily. B) Blood ethanol concentration was measured from mice treated with the two-bottle choice test (after the final day of 12%) or from mice fed a chronic liquid diet of 5% alcohol for four weeks (the Lieber-deCarli (LdC) chronic model). C) Serum concentration of liver enzymes alanine and aspartate aminotransferase (ALT and AST, respectively) were measured from mice from the two-bottle choice test and chronic LdC feeding model. D) Livers from mice in the two-bottle choice test or the chronic LdC feeding were stained with hematoxylin and eosin to observe lipid droplet accumulation and hepatocyte ballooning. n=3-6 mice/group. * p-value < 0.05 determined by student's *t*-test; error bars depict SEM.
NLRP3 inflammasome inhibition with MCC950 reduces alcohol consumption in female mice

Previously, we have shown the importance of the NLRP3 inflammasome and its role in alcoholic liver disease pathology\textsuperscript{70,113} as well as in neuroinflammation\textsuperscript{162}. Here, we hypothesize that signaling through the inflammasome may contribute to alcohol consumption and addiction. Therefore, we treated mice with daily \textit{i.p.} injections of the NLRP3 inhibitor, MCC950. Control mice were injected daily with an equal volume of the vehicle. We found that female mice treated with MCC950 consistently consumed less alcohol per kg body weight (Fig 5.2A) and had significantly lower alcohol preference (Fig 5.2C) compared to vehicle-treated controls. However, male mice were unaffected by MCC950 treatment in total alcohol consumption and preference (Fig 5.2B, D).
Figure 5.2 Female but not male mice treated with NLRP3 inflammasome inhibitor MCC950 consume less alcohol. Mice were housed singly and received daily intraperitoneal injection of either the NLRP3 inflammasome inhibitor MCC950 (5mg/kg BW) or a control saline injection. Two-bottle choice of water or alcohol in drinking water at increasing concentrations (3%, 6%, 9% and 12%) for four days each was provided ad libitum. Consumption was measured and mice were provided fresh water and alcohol daily. A-B) Dose of alcohol consumed at each concentration was normalized to mouse body weight for male and female mice. C-D) Alcohol preference was determined as a ratio of the volume of alcohol consumed to the total liquid volume consumed per day in male and female mice. n=6 mice/group. * p-value < 0.05 determined by student’s t-test; error bars depict SEM.
VX765, a Caspase-1 inhibitor, mildly attenuates alcohol consumption

To target the catalytic component of the NLRP3 inflammasome, Caspase-1, we used the inhibitor VX765. Daily VX765 treatment reduced alcohol consumption and alcohol preference in female mice when provided at 3 and 6%, but not at higher concentrations (Fig 5.3A, C). Alcohol consumption and preference at all four concentrations were unaffected in male mice treated with the Casp1 inhibitor, VX765 (Fig 5.3B, D).
Figure 5.3 Caspase-1 inhibition with VX765 mildly reduces alcohol consumption. Mice were housed singly and received daily treatment with either a Caspase-1 inhibitor VX765 (20mg/kg BW) or a control saline injection. Two-bottle choice of water or alcohol in drinking water at various concentrations (3%, 6%, 9% and 12%) for four days each was provided *ad libitum*. Consumption was measured and mice were provided fresh water and alcohol daily. A-B) Dose of alcohol consumed at each concentration was normalized to mouse body weight for male and female mice. C-D) Alcohol preference was determined as a ratio of the volume of alcohol consumed to the total liquid volume consumed per day in male and female mice. NB: Saline/DMSO data replotted from Figure 5.2. n=6 mice/group. * p-value < 0.05 determined by student’s t-test; error bars depict SEM.
Treatment with Anakinra reduces alcohol consumption both in female and male mice

Secretion of the effector molecules IL-1\(\beta\) and IL-18 is the ultimate step of inflammasome activation. We have previously highlighted the important role of IL-1\(\beta\) in alcohol-induced liver disease\(^{111,112}\) and neuroinflammation\(^{162}\). Anakinra (recombinant IL-1ra) is currently in clinical trials for numerous diseases including severe acute alcoholic hepatitis (NCT01809132). We found that female mice treated with Anakinra consumed less alcohol and had lower alcohol preference than vehicle treated animals (Fig 5.4A, C). Male mice also showed a modest trend toward reduced alcohol consumption at 3, 6, and 12% and statistically lower consumption at 9%, but no change in preference (Fig 5.4B, D). These results indicate that inhibition of IL-1 receptor signaling with the IL-1ra, Anakinra, can attenuate alcohol consumption in both genders in mice.
Figure 5.4 Recombinant IL-1r antagonist Anakinra reduces alcohol consumption in both female and male mice. Mice were housed singly and received daily treatment with either a recombinant IL-1 receptor antagonist Anakinra (25mg/kg BW) or a control saline injection. Two-bottle choice of water or alcohol in drinking water at various concentrations (3%, 6%, 9% and 12%) for four days each was provided ad libitum. Consumption was measured and mice were provided fresh water and alcohol daily. A-B) Dose of alcohol consumed at each concentration was normalized to mouse body weight for male and female mice. C-D) Alcohol preference was determined as a ratio of the volume of alcohol consumed to the total liquid volume consumed per day in male and female mice. NB: Saline/DMSO data replotted from Figure 5.2. n=6 mice/group. * p-value < 0.05 determined by student’s t-test; error bars depict SEM.
Consumption of sweet and bitter tastants is not reduced by inflammasome cascade inhibitor treatments

In order to test if inhibitor treatment had an effect on consumption or preference based on taste or other factors, we provided two alternate non-alcoholic tastants in a follow up experiment. Female and male mice were rested after the conclusion of the last 12% alcohol exposure for 30 days and were provided normal water only without any injections. Some mice were then provided a two-bottle choice of either water or a sweet tastant, saccharin (0.033% and 0.066%), in drinking water for four days at each concentration. The remaining mice were provided a bitter tastant, quinine (0.03 and 0.06mM) for the same duration. Mice that had previously received daily inhibitor or vehicle injections again received the same injections throughout the tastant test.

Although inhibitor treatments reduced alcohol consumption and preference, particularly in female mice, treatments did not reduce consumption of saccharin (Fig 5.5A-B) or quinine (Fig 5.5C-D) in either female or male mice compared with vehicle treatment. These results suggest that alcohol consumption is unlikely to be influenced by taste.
Figure 5.5 Consumption of non-alcoholic tastants (saccharin and quinine) does not indicate alterations in taste lead to reduced alcohol consumption in mice treated with inhibitors. A-B) Female and male mice were provided two-bottle choice between drinking water and saccharin in drinking water at two doses (0.033 and 0.066%) for four days each. C-D) A different cohort of female and male mice were provided two-bottle choice between drinking water and quinine in drinking water at two doses (0.03 and 0.06mM) for four days each. In all cases (A-D), some mice received daily treatment with Anakinra (25mg/kg BW), MCC950 (5mg/kg BW), VX765 (20mg/kg BW) or a control injection. n=3 mice/group. * p-value < 0.05 determined by student’s t-test; error bars depict SEM.
Vehicle injection modestly effects alcohol consumption

To investigate whether the stress of daily handling and injection impacted alcohol consumption, we compared mice injected daily with the vehicle to mice that were not injected and not regularly handled. Daily injection of vehicle modestly increased alcohol consumption in female mice at the lower concentrations of 3 and 6%, but not at higher concentrations (Fig 5.6A). Male mice drank slightly less alcohol at 3 and 6%, but not at higher concentrations (Fig 5.6B). This important observation suggests that, particularly in female mice who were most impacted by inflammasome inhibition, daily handling and injecting did not in and of itself reduce alcohol consumption.
Figure 5.6 Daily injection has modest effect on male and female mice alcohol consumption. Two-bottle choice of water or alcohol in drinking water at various concentrations (3%, 6%, 9% and 12%) for four days each was provided *ad libitum*. Consumption was measured and mice were provided fresh water and alcohol daily. Some mice were handled daily and received injection of vehicle while other mice were untreated. A-B) Dose of alcohol consumed at each concentration was normalized to mouse body weight for female and male mice. n=6 mice/group. * p-value < 0.05 determined by student’s *t*-test; error bars depict SEM. NB: Vehicle data replotted from Figure 5.2.
Discussion

The molecular regulatory pathways that promote alcohol consumption and preference are only partially understood. While most studies focused on neuromodulators and neuronal functions, recent evidence suggests a potential role for inflammatory mediators in alcohol addiction\textsuperscript{16,253}. Based on previous work revealing the importance of the NLRP3 inflammasome in alcohol-related pathology as well as early evidence that inhibiting immune signaling can affect alcohol consumption, in this study we hypothesized that inhibiting the inflammasome would reduce alcohol consumption in mice. Using the two-bottle choice paradigm, we show that female mice consume significantly greater amounts of alcohol compared to male mice. This indicates a significant gender difference in alcohol preference. Unlike the chronic 5\% alcohol in Lieber-DeCarli liquid diet model, our results reveal that although a two-bottle choice test is useful to study alcohol consumption and mouse behavior, it does not replicate the liver pathology associated with chronic alcohol consumption. We found that female, but not male, mice reduce their alcohol consumption and preference when treated with the NLRP3 inflammasome inhibitor MCC950, the caspase-1 inhibitor VX765, or with IL-1ra Anakinra. These findings emphasize the utility of the two-bottle test for behavioral assessments and provide novel insights into the role of the inflammasome pathway in alcohol use disorder and addiction.

Multiple alcohol consumption models exist for rodent experiments and each provides utility depending on the questions being tested. For example, the chronic 5\% alcohol in Lieber-DeCarli liquid diet model induces liver pathology including steatosis,
hepatocyte damage and mild inflammation as well as extrahepatic pathology including neuroinflammation. Our previous research showed that mice deficient in either ASC, Casp1 or IL-1r were protected from liver pathology in the chronic alcohol feeding model. We found that administration of Anakinra in the chronic alcohol feeding model effectively attenuated alcohol-induced liver disease and neuroinflammation in mice. Here, we chose to use pharmacologic inhibitors with therapeutic potential to show that targeting this same pathway significantly reduces alcohol consumption in female mice. Future behavioral assays using mice deficient in inflammasome components, and particularly cell-specific deletion of inflammasome component signaling within the CNS and periphery, may yield supporting evidence for targeting this pathway in human patients with AUD and provide insight into the critical cellular sources of inflammasome signaling that contribute to alcohol drinking behavior.

Interestingly, our previous studies focused on female mice in order to study liver disease because females have been shown to have greater sensitivity to alcohol and develop more severe alcohol-induced liver pathology than males. Given the differential effect of the inflammasome inhibitors in female and male animals on alcohol consumption and preference, it will be interesting to examine the effect of genetic knockout of the inflammasome in chronic feeding models on liver damage and inflammation in male mice as well. While the gut-liver axis is well established in alcoholic liver disease and alcohol-related liver inflammation as a result of alcohol-induced gut leakiness, communication between the intestine, liver and brain, which may influence alcohol consumption, continues to be an active area of research. Alcohol
consumption in the chronic Lieber-DeCarli 5% alcohol feeding model is often unaffected by genotype or treatment, likely due to the fact that this model delivers alcohol along with a liquid food diet so all calorie intake includes alcohol consumption. For this reason, the two-bottle choice provides an important model to measure behavioral changes that cannot be adequately assessed in other models of AUD.

Targeting inflammatory pathways has previously been shown to modulate alcohol consumption. Blednov et al. showed that alcohol consumption and preference were reduced in mice lacking functional inflammation-related genes such as CD14 and IL-6\textsuperscript{16}. Harris et al.\textsuperscript{359} recently showed that TLR4 manipulation in mice and rats had only modest effects on alcohol consumption, however, female TLR4 knockout rats had reduced alcohol preference compared to wild-type animals. These observations are consistent with our finding that inhibition of the inflammatory cytokine circuit attenuates alcohol consumption. Interestingly, IL-1ra knockout mice exhibit reduced alcohol preference in female and male mice, with female mice showing a stronger alcohol-aversive behavior\textsuperscript{16}. It is interesting that both IL-1ra knockout mice\textsuperscript{16} and mice treated with Anakinra in this present study (recombinant IL-1ra) would exhibit similar alcohol-aversive behavior. It is possible that the total body knockout of IL-1ra throughout development compared with the temporally-limited \textit{i.p.} dosing of Anakinra could induce different behavioral outcomes. It may also be the case that systemic administration of concentrated Anakinra, which has been shown to cross the blood-brain barrier, has an effect different from that of knocking out physiologically normal IL-1ra expression\textsuperscript{360}. Indeed, Anakinra has previously been shown to be effective at reducing alcohol-induced sedation and
improving recovery from alcohol-induced motor impairment in mice. Further study comparing IL-1ra knockout mice and pharmacologic treatment with recombinant IL-1ra will be necessary to further dissect these findings.

The link between immune signaling and a behavioral response to alcohol exposure was highlighted in a study by Blednov et al. LPS, a potent TLR4 ligand naturally found on the surface of gram-negative bacteria, was injected in mice who were then provided access to alcohol. LPS injection produced a prolonged alcohol preference and mice deficient in CD14, an important component of the TLR4 receptor complex that recognizes LPS, were protected from this preference induction. In the same study, investigators examined firing rates of dopaminergic neurons in the ventral tegmental region of the brain, a key area in the reward pathway. LPS injection reduced firing rates in these neurons which could lead to an increased threshold for a rewarding dopamine response. These experiments tie together TLR4 immune signaling, neurochemical reward pathways and alcohol consumption.

Our present study adds insight into a parallel pathway, the NLRP3 inflammasome, that also influences alcohol preference. Inhibition of NLRP3 inflammasome activation prevents production of IL-1β that is a central amplifier of pro-inflammatory responses and cytokine production. Unlike other pro-inflammatory cytokines, TNFα, IL-6 or CCL2, that are induced by a single danger signal through NF-kB activation, IL-1β production requires a secondary danger signal and inflammasome activation. This unique regulatory feature of IL-1 highlights the importance of inflammasome activation in alcohol consumption and preference. Indeed, a recent study
showed that blockade of IL-1β signaling in the ventral tegmental area (VTA), a critical area in the mesolimbic reward circuitry, reduced dopamine release in response to cocaine exposure\textsuperscript{363}, which may contribute to addiction and seeking more reward from a given drug. While the exact signaling mechanism involved has not been fully described, these data highlight an important role for IL-1β and immune signaling in addiction.

The different effects of inflammasome inhibition in females versus male mice were notable in our present study. However, sex difference related to alcohol-induced pathology are not unexpected, as ample evidence exist suggesting that males and females respond differently to alcohol exposure. Indeed, women have a stronger immune response and more prominent organ damage than males after alcohol consumption\textsuperscript{356,358,364}. Neurotoxicity and immune activation is greater in female mice as well\textsuperscript{365}. Given these sex differences, perhaps it is not surprising to observe differential behavior in male and female animals when treated with immune inhibitors. Knowledge of these differences may be critical to development of future therapeutics for alcohol use disorder and may be important in developing more personalized medicine approaches to treating addiction and disease.

Our observations further highlight the potential significance of inflammation pathways in alcohol addiction and provide evidence for new therapeutic interventions to attenuate alcohol preference. It remains to be evaluated whether these gender differences and/or inflammasome pathways play a role in alcohol preference in humans. The need for more effective therapies to both curb alcohol consumption and treat alcoholic liver disease remains high. Medications that can both reduce consumption and alleviate some
of the organ pathology associated with chronic intake would be a significant advancement for the field of alcohol treatment. Targeting the inflammasome may serve this purpose and further research will be needed to show the efficacy of blocking organ disease and reducing consumption.
Conclusion

Here, we show that blocking the NLRP3 inflammasome at multiple points reduces alcohol consumption and preference in female but not in male mice. We also show that while the two-bottle choice test may be useful as a behavioral assay, it does not induce pathology typically associated with alcohol use disorder. Our novel findings add new insights into the role of the inflammasome and offer promising new targets for treating alcohol use disorder.
CHAPTER VI
Discussion

Introduction

The simplicity with which alcohol can be consumed and enjoyed, whether in social settings, communal meals or any of the varied occasions in which alcohol-containing beverages are consumed, is hardly comparable to the complicated inter-organ process that occurs after alcohol flows into our bodies. The normal physiology involved with casual alcohol consumption involves an axis of organs including the intestine and microorganisms of the gut microbiome, the liver, the immune system and the central nervous system (CNS). Overconsumption of alcohol, such as occurs in alcohol use disorder (AUD), is associated with even more complicated inter-organ communication and pathophysiology that leads to organ dysfunction, adaptation and damage. Research suggests that some of the organ dysfunction and adaptation, particularly in the CNS, can reinforce aspects of alcohol consumption and lead to addiction, thus initiating a vicious cycle of alcohol consumption, continued dysfunction and adaptation, followed by more consumption. Efforts to better understand the physiology and pathophysiology of alcohol consumption and identify areas for therapeutic intervention are critical to helping the millions world-wide who suffer from AUD and its associated medical sequelae.

The previous chapters provide novel evidence that alcohol consumption induces organ-specific immune activation and pathology between the intestine, liver, peripheral immune system and the central nervous system. After consumption, alcohol encounters
the microorganisms of the intestine and causes changes in the flora of the gut that is associated with proinflammatory signaling in the intestine itself. Bacterial content of the intestine significantly contributes to features of alcoholic hepatitis, including steatosis and inflammation, in the liver and neuroinflammation in the brain and reduction in the intestinal bacterial load with antibiotics protected the liver and CNS from these changes. Immune signaling and the peripheral immune system are critical players in alcohol-induced organ pathology and, through blockade of CCR2/5 signaling, we have shown that peripheral macrophage infiltration into the liver and the CNS significantly contribute to this process. We demonstrate that chronic alcohol consumption leads to a dysregulation in excitatory and inhibitory synapses that is associated with upregulation of complement proteins and activated microglia in the CNS. Finally, we have established that immune signaling, through the NLRP3 inflammasome, has significant influence over behavior associated with alcohol seeking and that inhibiting inflammasome activation can reduce alcohol consumption in female mice. Taken together, these studies provide novel therapeutic approaches, including blockade of CCR2/5 chemotaxis and inhibition of the inflammasome, to protect from the complicated interactions between the gut, liver, peripheral immune system and the brain stimulated by alcohol consumption.

The intestinal microbiome is critical in the development of alcohol-induced liver and CNS inflammation

We analyzed the intestinal microbiome from the cecum of mice treated with a relatively short alcohol treatment (ten days) with one acute binge. This is a model that has
been useful because of its replication in mice of human alcoholic hepatitis, particularly steatosis, liver enzyme elevation and neutrophil-associated hepatic inflammation\textsuperscript{110,259}. The intestinal microbiome has not previously been studied in this model and expanding our knowledge of alcohol-associated changes in the microorganism profile, with the advent of techniques for bacterial taxonomic analysis and the increasing recognition of the microbiome in disease, is important.

We chose to suppress the intestinal microbiome in order to study the microbiome’s function. Others have instead sought to alter the microbiome profile, using prebiotics, probiotics or direct administration of particular bacteria, to study alcohol-microbiome-liver interactions. One such study is particularly relevant to the data presented in Chapter II. Grander et al.\textsuperscript{281} observed that alcohol reduced \textit{Akkermansia} in human patients with alcoholic hepatitis, similar to the findings presented here and observed by others\textsuperscript{45}. Replenishing \textit{Akkermansia} both prevented and ameliorated alcohol-induced liver injury, steatosis and immune cell infiltration\textsuperscript{281}. Future studies are likely to explore potential benefits of replenishing bacterial communities, such as \textit{Akkermansia}\textsuperscript{272} that are reduced in the alcohol-exposed microbiome, or the potential harmful effects of other bacteria (such as those that are increased in the alcohol-exposed intestinal microbiome) either in the presence of or independent from alcohol consumption in order to expand our understanding of microbiome function in ALD. While specific metabolites of particular bacteria (such as \textit{Akkermansia}) have not been well-characterized or studied \textit{in vivo}, evidence is already emerging that some general bacterial metabolites, including short-chain fatty acids, can be neuroactive and influence microglial development,
function and morphology. The development of targeted anti- or pro-biotics that could promote or suppress bacterial communities specifically may be of significant benefit in the future, although the use of broad-spectrum antibiotics, like those used in Chapter II, would not be an appropriate approach to treating or protecting from ALD or alcohol-induced neuroinflammation due to the significant risk of antibiotic resistance as well as the dangers associated with a depleted intestinal microbiome (i.e. filling that niche with 
\textit{clostridium difficile} or other dangerous pathogenic microbes). Broadening the scope of future studies using probiotics or promoting a particular microorganism community, and including investigations into the CNS-related effects, will be critical.

Treatment with antibiotics in this study significantly reduced culturable stool bacteria, 16S bacterial DNA and serum lipopolysaccharide (LPS), all indicating that the intestinal microbiome was significantly suppressed. Previous studies have also used antibiotics to examine the role of the gut microbiome in ALD and have observed protection from features of ALD similar to our results. However, these studies used an antibiotic cocktails of nonabsorbable antibiotics whereas we used absorbable antibiotics, including ampicillin, metronidazole and vancomycin, along with neomycin which is non-absorbable. It is possible that there were off-target effects of absorbable antibiotics. One such possible side effect could be the interaction of metronidazole with the liver and alcohol metabolism, as drinking alcohol is contraindicated in human patients taking metronidazole. In our study, both alcohol-fed mice treated with metronidazole and those without antibiotic treatment had similarly elevated liver enzymes as those with antibiotic-treatment, suggesting the absence of any potential liver-related toxicity.
Additionally, we observed a general improvement in liver histology (steatosis, immune cell infiltration) and proinflammatory cytokine expression indicative of protection by antibiotic treatment and intestinal bacterial load suppression.

Two alternative approaches to examine the effect of the gut microbiome on neuroinflammation would include the use of non-absorbable oral antibiotics or germ-free mice. Non-absorbable antibiotics would restrict the bacterial killing effect to the intestinal lumen and could eliminate off-target effects of the medications or extra-enteric antimicrobial activity. Some antibiotics, such as the tetracycline-derivative minocycline, has been used as an anti-inflammatory agent can suppress microglial reactivity to various stimuli. While the antibiotics used in this study are not tetracycline derivatives, the possibility of absorption and systemic effects in the body has not been ruled out. Using non-absorbable antibiotics would limit such an “off-target” effect (i.e. not restricted to the reducing the intestinal microbiome). Additionally, the use of germ-free mice would remove the need for pharmacologic treatment altogether. However, this latter approach has its own limitations. Treatment of germ-free mice with alcohol has been shown to induce rather than reduce significant liver damage including liver enzyme elevation, histologic changes and significantly elevated levels of inflammatory markers. The discrepancy between studies that use antibiotics, including ours in Chapter II, and those using germ-free mice is likely accounted for by the role played by the microbiome in immune development throughout life.

Germ-free mice could, however, provide a number of interesting avenues to study the role of the microbiome in alcohol-induced steatohepatitis and neuroinflammation.
Microbiome transfers into germ-free animals followed by alcohol exposure could help differentiate the role of developmental exposure to commensal bacteria and the microbiome present at the time of alcohol exposure. Indeed, germ-free animals that receive gut microbiome transfer from human patients with ALD exhibit signs of steatohepatitis, even without exposure to alcohol\textsuperscript{69}. To date, no study has explored the effects of alcohol on neuroinflammation in germ-free animals or in germ-free animals that receive intestinal microbiome transfer from alcohol-exposed individuals and further exploration using these or other approaches will critically expand our understanding of the gut microbiome’s influence on alcohol-induced CNS changes.

Chapter II provides evidence of protection from intestinal, hepatic and CNS inflammation in mice with suppressed gut microbiome after alcohol consumption. Although this approach provides insight into the overall role of the microbiome itself on multiple organs, it is limited by a lack of organ-specific blockade of microbiome signaling. Additionally, we and others\textsuperscript{29,80,88} have shown that systemic inflammation is induced by alcohol consumption. The presence of systemic inflammatory signals, initiated by leakage of bacterial products from the gut\textsuperscript{24,26,31,32}, could be responsible for the communication between, for example, the liver and brain leading to neuroinflammation and CNS changes. Alternative approaches will be needed to address whether any one organ (intestine, liver, brain or others) is more important in initiating alcohol-induced inflammation or if each organ is affected in unique ways. One way to address this question would be to specifically target certain immune receptors, such as Toll-like receptors (TLRs), that are responsible for sensing various bacterial components.
For example, TLR2 recognizes gram-positive bacterial surface molecules and TLR4 is the main surface receptor on immune cells for recognition of gram-negative LPS\textsuperscript{167,367}. Whether through Cre/Lox genetic targeting\textsuperscript{368}, AAV delivered silencing\textsuperscript{369} or other mechanisms, knocking out or silencing TLRs in cell-specific immune compartments throughout the body (including in intestinal immune cells, circulating immune cells, liver Kupffer cells, CNS microglia and BBB juxta-vascular cells, etc.) would provide insight into the contribution of each organ and each cell-type to alcohol-induced dysfunction and inflammation. Previous research showing protection from ALD and neuroinflammation using TLR2 and TLR4 knockout mice has been mostly limited to whole-body knockouts\textsuperscript{170-172,280}. Exploring cell-specific immune responses will be particularly satisfying to answer important questions surrounding CNS alterations by alcohol and could provide novel approaches for AUD and addiction therapy.

**Chronic alcohol induces liver damage and inflammation that is dependent on CCR2/5 signaling**

Expression of the CCR2/5 signaling axis is upregulated in patients with ALD along with elevated macrophage and T cell markers in the liver. We therefore tested the hypothesis that signaling through CCR2/5, leading to leukocyte infiltration, is important in the development of chronic alcohol-induced liver damage, steatosis and inflammation. Use of the small molecule inhibitor Cenicriviroc (CVC) was hepatoprotective and reduced ALT increase, liver steatosis and fibrosis, infiltration of peripheral macrophages and proinflammatory expression, all of which had been induced in untreated alcohol-fed
mice. These findings are particularly promising as CVC is currently undergoing clinical trials and has the potential to be a beneficial therapeutic for patients with ALD.

Although inflammation is a protective physiologic response to acute insults, chronic inflammation has been implicated in multiple disease states and is associated with unhealthy dysfunction. In human patients with ALD, circulating monocytes are primed to respond with enhanced proinflammatory cytokine release upon immune challenge. Mouse models of chronic ALD induce infiltration of peripherally derived monocytes into the liver, as we have shown here. These infiltrated cells have subpopulations of infiltrating macrophages (IMs) that can be characterized as either tissue-protective and anti-inflammatory (high expression of phagocytosis-related genes, growth factors or tissue repair genes) or proinflammatory (elevated cytokines, chemokines and receptors). Interestingly, Wang et al. found significantly increased CCR2 expression in proinflammatory Ly6C<sup>hi</sup> IMs compared to the tissue-protective Ly6C<sup>lo</sup> IMs. Although we did not interrogate Ly6C expression in our model, it is possible that CVC (a CCR2 inhibitor) blocked the infiltration specifically of CCR2<sup>hi</sup> expressing Ly6C<sup>hi</sup> proinflammatory macrophages. Specifically targeting proinflammatory macrophage infiltration may prove to have additional benefits beyond CCR2/5 inhibition, which successfully protected the liver from alcohol-induced pathology.

Previous studies have attempted to elucidate the importance of macrophages in the liver, including both Kupffer cells as well as peripherally-derived macrophages. Using gadolinium chloride to deplete macrophages, Koop et al. showed significant protection from alcohol-induced steatosis, necrosis and inflammation. Our lab
previously depleted macrophages using clodronate and replenished the bone-marrow niche with cells deficient in caspase-1. Subsequent alcohol administration resulted in reduced liver damage compared to caspase-1 proficient animals, highlighting the importance of proinflammatory macrophage signaling in ALD. However, other forms of liver disease, including acetaminophen toxicity and fibrosis, are significantly worsened by elimination of macrophages, including Kupffer cells. Targeted depletion of resident Kupffer cells followed by alcohol administration would provide an interesting additional test of the role of resident versus peripheral macrophage in ALD.

In addition to targeting macrophage chemotaxis, genetic interruption of CCL2/CCR2 signaling has also proven beneficial in mouse models of ALD. In particular, livers exhibited less steatosis in CCL2 KO mice compared with CCR2 deficient animals. CVC reduced liver steatosis as well, providing further evidence of a link between CCL2 signaling and lipid accumulation in ALD. Experiments with isolated murine hepatocytes in vitro showed that CCL2 signaling can induce genes related to lipogenesis. These studies suggest that non-immune mechanism may, at least in part, be responsible for the reduction in liver steatosis.

**Alcohol-induced neuroinflammation is associated with peripheral monocyte infiltration through CCR2/5 signaling**

Here, we studied the role CCR2/5 signaling using a small molecule inhibitor and show that treatment reduced alcohol-induced macrophage infiltration to the brain, expression of some proinflammatory genes and proteins and altered the microglial
morphology changes and protein expression profile. These data provide novel insight into the mechanism by which IMs are attracted to the CNS as well as their impact on resident microglia after chronic alcohol consumption.

This pharmacologic approach does not differentiate cell-specific CCR2/5 blockade because Kupffer cells, hepatocytes, microglia, neurons and other cell types, in addition to circulating immune cells, also express CCR2 and CCR5. An interesting follow up study would be to establish genetic models that specifically knockout chemokine receptors in various cell types to explore the role of chemokine/receptor signaling both in peripheral and resident immune cells and in parenchymal cells. Testing CCR2/5 deficiency specifically in peripheral immune cells would shed significant light on their infiltration and role in organ inflammation after alcohol consumption.

Another question explored in this study was the role of infiltrating immune cells in the CNS after alcohol consumption with blockade of these macrophages using CVC. Alcohol-induced infiltration of peripheral immune cells has long been studied in the liver, but is only recently being recognized in the CNS and will certainly require further exploration. Here, we report that infiltration of macrophages preferentially occurred in the hippocampus with a roughly two-fold increase in cells expressing CCR2 in the hippocampus. The hippocampus is important in memory and learning and alcohol has been shown to influence this area in particular to. However, the role of infiltrating macrophages in this CNS region deserves further exploration and we have only begun to understand how these cells may contribute to local proinflammatory gene expression. Further study using CVC along with memory
tasks in alcohol-exposed mice will be an important follow up to the present study. Additionally, studying the important role played by resident macrophages such as Kupffer cells (discussed above) and microglia will also yield critical insight into innate immune signaling in AUD.

Recent identification of a CSF1R inhibitor PLX5622 has established a model for studying what happens in the CNS when microglia are removed (or at least dramatically reduced in number). Indeed, one study exists so far exploring how alcohol affects the microglia-deficient CNS. Walter et al. treated with PLX5622 followed by acute alcohol administration (6g/kg binge) and observed a reduction in proinflammatory cytokine expression including Tnfα and Ccl2 compared to non-PLX5622 treated mice. There are many caveats that accompany the depletion of microglia, including whether any cell types (peripheral macrophages, immature microglia or other glia) fill the niche and alter their own behavior as well as whether the process of microglial depletion (clearance of debris, released cellular content) impacts the surrounding CNS, among others. These will most certainly be explored with the continued use of anti-microglial compounds such as CSF1R inhibitors. Regardless, further exploration using the CSF1R inhibitor or other methods to transiently deplete microglial in other models of AUD (including chronic alcohol models similar to those described here and elsewhere in animals), will help to answer important questions. These may include: what role do microglia play in proinflammatory cytokine and chemokine production after alcohol exposure? Do microglia serve a protective role or more harmful role and how does the time course of alcohol exposure (acute versus chronic) influence these functions? Are there “behavioral”
changes (addiction, memory impairment, social deficits) induced or reduced by alcohol in
the absence of microglia? How are alcohol-induced synapse changes impacted in the
absence of microglia? These and many more questions deserve further investigation to
advance our understanding of the neurobiology underlying AUD.

Inhibiting inflammation signaling can reduce alcohol consumption in rodents. As
we show in Chapter V, blockade of inflammasome activation using pharmacologic
methods in mice reduced alcohol consumption and preference in the two-bottle choice
model. Others have studied both genetic and pharmacologic approaches to block various
immune pathways and inhibit alcohol-seeking behaviors in animals. Expanding our
current understanding of the role of CCR2/5 signaling with these behavioral studies can
be accomplished using CVC or similar targeted therapies. A possible experimental
approach would include replicating the two-bottle choice or using other addiction models
including the drinking in the dark model, along with CVC administration. Blednov et al.
observed that mice deficient in CCR2, CCL2 and CCL3 (both chemokine ligands for
CCR2) consumed less alcohol in the two-bottle choice test and displayed stronger
conditioned taste aversion. Like our observations with inflammasome inhibitors
(Chapter V), sex differences were observed in that study and only female CCL2 deficient
mice consumed less alcohol; male mice behaved similarly to wild-type animals.
However, these studies are in mice with total body receptor deficiencies since birth and a
more clinically relevant approach using pharmacologic CCR2/5 inhibition is needed.
Valenta et al. recently explored CNS infusion of CCL2 and showed that alcohol
consumption was associated with CCL2 administration in a dose-dependent manner,
providing additional evidence for a connection between CCL2/CCR2 signaling and alcohol-related behavior\textsuperscript{213}.

**Chronic alcohol alters excitatory and inhibitory hippocampal synapse density**

Chapter IV describes increases in the expression of hippocampal presynaptic excitatory protein VGLUT1 along with postsynaptic excitatory protein HOMER1 and an increase in colocalization of these proteins suggestive of increased synapses in alcohol-fed mice compared with pair-fed controls. Additionally, expression of the inhibitory postsynaptic protein Gephyrin was increased in the CA1 and CA3 region of the hippocampus and colocalized inhibitory synapses (VGAT/Gephyrin) were increased in the CA3 in alcohol-fed mice. Coincidentally, complement proteins C1q and C3 were also elevated in the hippocampus alongside the increases in excitatory and inhibitory synapses.

Synapse pruning occurs throughout the brain during development and involves microglia responding to complement-tagged synapses marked for engulfment and degradation\textsuperscript{157-159}. Therefore, our observations of increased complement expression along with increased synapses is somewhat surprising based on our current understanding of complement-dependent synapse pruning. However, complement proteins are also involved in inflammatory signaling. In our model of alcohol consumption, we observe significant upregulation by alcohol of inflammatory genes and proteins in the CNS and particularly in the hippocampus. We also observe morphological and surface marker changes in microglia consistent with an activation phenotype. Therefore, although
synaptic pruning by microglia occurs via complement signaling in homeostatic
development, dysregulation of microglia, complement expression and even neuronal
synapse activity occur after chronic alcohol exposure. This dysregulation could impact
the normal physiological process of pruning and may lead to altered synaptic engulfment,
despite an increase in complement protein colocalization with synapses. Additionally,
complement expression may be primarily caused by the proinflammatory milieu
associated with alcohol-induced neuroinflammation and therefore not serve a synaptic-
regulatory purpose but rather an inflammatory role. This may point toward changes in
microglial responsiveness to synapse regulation, perhaps due to a preoccupation with
other inflammatory signaling in the CNS caused by alcohol consumption, and certainly
deserves further investigation.

Using a model of intermittent binge alcohol administration in adolescent rats,
Risher and colleagues139 have provided some insight into mechanisms that may be
involved in alcohol-related synaptic dysregulation. In these studies, the authors describe
an initial decrease in colocalization of excitatory synaptic markers VGLUT1 and PSD95
in the hippocampus after four alcohol binges (administered by intragastric gavage).
However, after six more gavages (ten days after the early time point), VGLUT1/PSD95
colocalization was increased in alcohol-exposed rats compared to control animals and
these changes were associated with correlated levels of thrombospondin proteins,
previously shown to be important in synaptogenesis and maintenance375,376. Interestingly,
in a separate study138, rats who were treated with intermittent adolescent alcohol binges
followed by four weeks of abstinence showed decreased excitatory synapses
(VGLUT1/PSD95 and VGLUT1/SAP102 colocalization) in the CA1 of the hippocampus. The researchers noted that the dendritic spines in adult rats who had been treated with adolescent alcohol were of a more immature morphology (long and thin filopodia) compared with control rat dendrites (stubby and mushroom morphology)\textsuperscript{138}. Additionally, binge alcohol administration in rats has been shown to increase dendritic spine densities coinciding with excitatory synaptic activity in the prefrontal cortex\textsuperscript{194}. Taken together, these two studies suggest that, at least in a model of adolescent binge exposure, alcohol acutely decreases hippocampal excitatory synapses which then rebound during chronic exposure, only to decrease again following abstinence.

While the time course of synaptic density changes may be dynamic (even convoluted) when factoring in abstinence and various binge-drinking models, Montesinos et al.\textsuperscript{195} also used a model of adolescent alcohol exposure and observed an upregulation of synapses (in the prefrontal cortex) when mice were sacrificed immediately after alcohol exposure, similar to the findings we present in Chapter IV. Further research will be needed in chronic models of alcohol and at different life stages to better determine the time course of synaptic dysregulation and involved mechanisms. However, these studies, as well as the data presented here, make clear that alcohol exposure influences synaptic densities in a dynamic way that may underlie such behavioral manifestations as addiction and cognitive dysfunction in patients with AUD.

Excitotoxicity is a mechanism of neuronal damage and degeneration caused by the excitatory neurotransmission. Following chronic alcohol use, a hyperexcitable state exists (caused by adaptive neurotransmitter receptor expression, including glutamate and
GABA receptors) in the CNS that can predispose patients with AUD to seizures if alcohol (which normally depresses excitation in the CNS) is removed\textsuperscript{377,378}. We and others now report increased expression of excitatory synapses in the CNS in animal models of alcohol consumption, despite the association of chronic alcohol. The increase in synapses we observe may be related to the timing of our experiments, as we only look at one time point. As discussed above, the increase in excitatory synapses, coupled with the neuroadaptive changes in neurotransmitter receptor expression in chronic alcohol, may lead to eventual excitotoxicity and neurodegeneration. However, the association of excitotoxicity with neuronal damage caused by alcohol remains an area of active debate\textsuperscript{379} and further research into the cause and effect will be needed to resolve this outstanding question.

Important questions remain related to changes in synaptic density following chronic alcohol. These include whether the observed changes are a product of dysregulated synaptogenesis, dysregulated pruning processes or dysregulated neuronal activity. Indeed microglia have been shown to be involved in both developmental synaptogenesis\textsuperscript{380} as well as pruning\textsuperscript{157}, although at a more advanced age their role in synaptic regulation is less clear. We and others have presented evidence of activation and inflammatory changes in microglia following alcohol\textsuperscript{142,171,187,188}. This alcohol-induced immune activation may interrupt normal pruning processes in microglia and lead to more (or even fewer) synapses, depending on the model and time point. However, the change in synaptic numbers may also be intrinsic to neuronal activity and synaptogenesis. Further work will be necessary to distinguish these possible mechanisms, including
consideration of other glia, such as astrocytes, that may also play a critical role in alcohol-related synaptogenesis and synapse regulation\textsuperscript{375}. As previously discussed, looking at various time points beyond six-week treatment of alcohol (including following a period of abstinence) will be important in understanding if the increase in synapses we describe here persist or if the effect of alcohol on hippocampal synapses is more dynamic.

**Inhibition of the NLRP3 inflammasome cascade reduces alcohol consumption in female mice**

In an effort to apply our observations that alcohol induces systemic, hepatic and CNS inflammation to a therapeutic model, we used anti-inflammatory NLRP3 inflammasome inhibitors in a mouse model to measure alcohol consumption and preference. We found that female mice consumed significantly less alcohol and exhibited less alcohol preference when treated with inflammasome inhibitors compared to control mice with limited effect in male mice. These findings are consistent with other studies that have used genetic and pharmacologic approaches to influence addiction behavior\textsuperscript{15,16,245,359} and establish the inflammasome as a novel target for further research and potential AUD treatment.

In order to validate our findings in the two-bottle choice test, it would be interesting to apply other behavioral assays, such as drinking in the dark or conditioned place preference/aversion, to alcohol-consuming mice treated with inflammasome inhibition. We observed behavior consistent with resistance to alcohol-seeking, a
component of addiction that can be influenced by both an aversion to negative effects of alcohol consumption and the positive influence of reward-pathway activation\textsuperscript{11}. Indeed, previous research has demonstrated that the response to alcohol of CNS dopamine regulation differs between females and males\textsuperscript{381}. Whereas female rats had increased dopamine release in the nucleus accumbens and striatum (part of the mesolimbic dopamine reward pathway), male rats had attenuated release in response to low levels of alcohol and reduced dopamine levels when exposed to high alcohol levels\textsuperscript{381}. Therefore, studying reward-associated dopaminergic signaling within the mesolimbic cortical system in alcohol-treated mice with or without inflammasome inhibitors or genetic knockout of inflammasome components would yield important insight regarding how immune signaling influences addictive behavior.

Because inflammasome-related behavior could be influenced by alcohol-induced signals that originate and act within the CNS or could derive from peripheral signals that communicate to the CNS, future studies may investigate cell-type specific alteration of the inflammasome pathway, for example, in peripheral immune cells (Kupffer cells, monocytes, etc.) or CNS inflammasome-expressing cells (microglia or astrocytes). In fact, with such modest alcohol consumed in the two-bottle choice test used in our study (limited elevation of BEC, no ALT/AST change observed), there is unlikely to be significant liver or CNS inflammation like that observed in our other chronic studies. This could suggest that signaling through the inflammasome at levels below our detection ability or within a very small population of cells is important for alcohol-seeking behavior. Longer-term models of two-bottle choice offer intermittent alcohol with
alcohol-free days in between. Studies after thirty days of alcohol (sixty days total) have revealed significant changes in microglia and astrocyte gene expression with significantly upregulated microglial immune signaling$^{189,190}$ and may be an interesting alternative model to investigate with inflammasome inhibition in order to achieve more significant inflammation.

Rather than inhibiting inflammasome signaling in an effort to ameliorate alcohol addiction, treating mice to activate the inflammasome or mimic its activation could also shed important light on the inflammasome in alcohol addictive behavior. This could be accomplished both with peripheral and central injection of proinflammatory molecules (such as LPS) or IL-1$\beta$, the active end-product of NLRP3 inflammasome activation. A similar paradigm with intracranial CCL2 injection was recently used and showed increased alcohol consumption when the chemokine was injected and this effect lasted for weeks after CCL2 infusion was terminated$^{213}$. Similar studies, including targets of the inflammasome signaling pathway, are warranted to explore what immune pathways are involved in the CNS biochemistry leading to alcohol consumption.

**Conclusion**

The data presented here span the body, much like ingested alcohol does after absorption. We sought to investigate the role of inter-organ cross-talk and common pathways of immune activation in the intestine, liver, peripheral circulation and the central nervous system. We describe changes in the gut microbiome that may contribute to organ dysfunction in the liver and brain associated with alcohol consumption. We
present novel evidence for therapeutic interventions, such as targeting the gut microbiome, CCR2/5 signaling and inflammasome signaling, that are beneficial in our various models of alcohol consumption and could prove more broadly applicable in human populations with alcohol use disorder. The data presented here are a testament to the complicated path alcohol takes in our bodies and the complex interactions among the gut-liver-immune-brain axis induced by alcohol.

To future explorations of interactions between alcohol and these systems, cheers!
References


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