IDENTIFYING MOLECULAR MECHANISMS IN ALCOHOL USE DISORDERS: TLR4 METHYLATION AND CYTOKINE EXPRESSION

by

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The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

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Identifying Molecular Mechanisms in Alcohol Use Disorders: TLR4 Methylation and Cytokine Expression

Thesis directed by Professor Kent E. Hutchison

Alcohol use disorders (AUDs) are associated with significant morbidity, mortality and socioeconomic costs in the United States. Despite decades of research, the best treatments are only modestly successful, in part due to the neurobiological complexity of AUDs. Although the molecular mechanism(s) driving the effects of alcohol on the brain and body are not fully understood, human and animal studies have converged to underscore the role of neuroinflammation. Alcohol increases inflammation via binding to Toll-like Receptor 4 (TLR4) receptors on immune cells. Chronic alcohol-induced, TLR4-mediated inflammatory signaling may lead to cellular damage. One hypothesized consequence of alcohol-induced immune signaling disruptions is neuronal cell death in frontal control regions of the brain, consistent with inhibitory deficits observed in AUD. This study explores inflammation as a molecular mechanism underlying cognitive deficits in AUD.

We collected *N*=82 subjects (mean age=29.91(*4.5*), range 25-40), including n=43 heavy drinkers and n=39 light drinkers. In addition to administering a battery of questionnaires measuring psychological variables, substance use and other health behaviors, we measured circulating and LPS-stimulated pro-inflammatory cytokines (IL-6, IL-8, IL-1β) and the damage-associated molecular pattern (DAMP) molecule HMGB1, TLR4 promoter methylation, and cognitive performance. We hypothesized that alcohol consumption would be associated with increased circulating and stimulated cytokines and HMGB1, greater TLR4 methylation and

lower cognitive performance. We further hypothesized that inflammatory cytokines would mediate the relationship between alcohol consumption and cognition.

Contrary to these hypotheses, no group differences emerged for inflammation or cognitive performance. When alcohol was examined as a continuous predictor, a significant relationship emerged between alcohol consumption and circulating and stimulated IL-6 and between cannabis use and circulating IL-1β. Follow-up analyses indicated that cannabis use moderates the relationship between alcohol and circulating IL-6, such that individuals who did not use cannabis in the 90 days prior to the study showed a strong correlation between alcohol and IL-6, whereas those who did use cannabis did not demonstrate any association. Future work should explore the interaction between alcohol and cannabis on peripheral inflammation, ideally with the inclusion of structural or functional brain imaging to examine how potential changes in peripheral inflammation impact neural control circuitry.

Dedication

This manuscript is dedicated with all my love, gratitude, and admiration, to my favorite psychologists, Linda Ruehlman and Paul Karoly, whom I am immeasurably lucky to have as parents. I attribute this accomplishment—as well as most other things—to their unwavering support for my passions and unrelenting faith in my abilities. It will be impossible to ever thank them enough, but I'll keep trying.

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INTRODUCTION

Alcohol use disorders (AUDs) represent a serious mental and physical health problem in the United States (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011; Stahre, Roeber, Kanny, Brewer, & Zhang, 2014). Specifically, AUDs contribute significantly to global morbidity and mortality (Lozano et al., 2012; Murray et al., 2012) and are one of the leading causes of disability worldwide (Whiteford et al., 2013). Although decades of basic science research and clinically oriented studies have shed light on the etiology and risk factors associated with AUD, the best pharmacological and behavioral treatments demonstrate only modest efficacy (Anton et al., 2006). Given the neurobiological complexity of AUD (Volkow & Baler, 2014), the development of more efficacious treatments will undoubtedly require gaining a deeper mechanistic understanding of the neural and molecular mechanisms that underlie the etiology and course of the disorder. Considerable research has characterized specific neural circuitry changes that occur in AUD, but less is known about the molecular bases for these deleterious alcohol-related neuroadaptations. The identification of molecular mechanisms that contribute to alcohol-induced neural alterations may eventually lead to promising new pharmacological treatment options.

Theoretical Models of Addiction

To elucidate molecular mechanisms that may be involved in developing AUD and promoting relapse, it is important to consider the typical neural and behavioral changes that occur over the course of the disorder. Over the past 75 years, research spanning such disciplines

as psychology, psychiatry, molecular biology, neuroscience and integrative physiology has leveraged innovative techniques such as neuroimaging, animal models, genetics and epigenetics to shed light on critical neuroadaptations characteristic of AUDs (e.g., Candon, Ward, & Pandina, 2014; Schuckit, 2014). Based on this body of work, theoretical models of addiction have emphasized the role of neural control and reward circuits in directly influencing substance use behavior (Karoly, Harlaar, & Hutchison, 2013; Koob & Volkow, 2010). Specifically, the reward network (Kalivas & Volkow, 2005) promotes the urge to use a substance, and the control network (Bechara, 2005) determines whether an individual will act upon such an urge (Hutchison, 2008). Over the course of addiction, there appears to be progressive dysfunction of these interconnected reward and control circuits, such that the reward network is increasingly "strengthened" while the control network is "weakened" (Baler & Volkow, 2006; Crews & Boettiger, 2009; Haber & Behrens, 2014; Karoly et al., 2013). Currently, the molecular mechanisms underlying the neural circuitry changes associated with alcohol dependence are not fully understood. The present study aims to explore molecular mechanisms that may underlie alcohol-related changes specifically within *control systems* in the brain.

Neural Control Systems and Alcohol Use Disorders

Evidence suggests that neurocognitive mechanisms of inhibitory-control are undermined in AUDs. Behaviorally, such control dysfunction may be evidenced by continued alcohol use despite negative psychosocial, medical, financial or other consequences. In the brain, cognitive control functions are subserved primarily by a network of frontal structures including the inferior frontal gyrus (IFG), orbitofrontal cortex (OFC) and dorsolateral prefrontal cortex (dlPFC) (Boettiger, Kelley, Mitchell, D'Esposito, & Fields, 2009; Claus, Kiehl, & Hutchison, 2011; Karoly et al., 2013). These areas underlie reflective cognition, control over impulsive decisions,

evaluation of the magnitude of potential rewards, and the urge to use substances (Bechara & Van Der Linden, 2005; Goldstein & Volkow, 2011; Wong et al., 2006).

Both animal and human studies have shown that these critical frontal brain regions are compromised in the context of chronic and/or heavy alcohol exposure (see Crews & Boettiger, 2009). Although alcohol dependent individuals have lower volumes of both cortical and subcortical brain structures (Crews & Nixon, 2009), the frontal lobes appear to incur the most alcohol-related damage (Rosenbloom, Sullivan, & Pfefferbaum, 2003; Sullivan & Pfefferbaum, 2005). Notably, the frontal cortex (Harper & Kril, 1989) and the orbitofrontal cortex (Miguel-Hidalgo, Overholser, Meltzer, Stockmeier, & Rajkowska, 2006) show significant neuronal loss in post-mortem studies of alcohol dependence.

Currently, the molecular mechanisms underlying alcohol-induced frontal lobe damage are not clearly understood, yet emergent research has highlighted the role of innate immune signaling and inflammation in promoting neural adaptations related to acute and chronic exposure to alcohol (Mayfield, Ferguson, & Harris, 2013). One hypothesis is that the deleterious neuro-inflammatory sequelae of alcohol use may contribute to neurodegeneration (Perry, Nicoll, & Holmes, 2010), and such damage may be particularly evident in frontal control areas of the brain (e.g., He & Crews, 2008; Qin & Crews, 2012). However, further research is needed to elucidate the relationship between alcohol, inflammation and damage within neural control circuitry.

Molecular Mechanisms Underlying Alcohol-Induced Adaptations in Neural Control Circuitry

Immune Signaling and TLR4. The human immune response involves both innate immunity, which occurs quickly as the first line of defense against toxins, and adaptive immunity, which

occurs later and is associated with immune memory. The focus here will be primarily on innate immune signaling. A critical role of innate immune cells is to respond to invaders or danger signals, typically causing inflammation, which is a localized response that generally involves heat, redness, swelling, pain, loss of function and cell migration. The innate immune system relies on germline-encoded, pattern-recognition receptors, such as Toll-like receptors (TLRs), to identify invading pathogens and activate various immune cells. Of particular importance to the discussion of alcohol and immune signaling is TLR4, a cell-surface receptor found in numerous peripheral immune cells, that recognizes multiple pathogen/danger-associated molecular patterns (P/DAMPS) associated with pathogens or cellular signals of danger or stress. Importantly, the gram-negative bacterial cell wall component lipopolysaccharide (LPS) is an endotoxin recognized by TLR4, and binding of LPS to TLR4 triggers signaling pathways (via the oxidant-sensitive pro-inflammatory transcription factor NF-kB) that ultimately lead to an increase in blood levels of pro-inflammatory cytokines (Crews, Zou, & Qin, 2011).

Peripheral cytokines, such as interleukins, can then influence the inflammatory response in the central nervous system (CNS; comprised of the brain and spinal cord in humans). The mechanism through which this occurs is not completely understood, but may involve the ability of certain cytokines to penetrate the blood brain barrier (BBB) via circumventricular organs or active transport across the BBB membrane (Quan & Banks, 2007).

Another DAMP molecule that binds to TLR4 is high mobility group box 1 protein (HMGB1), which is a chromatin protein that regulates gene transcription, and is produced by immune cells in response to a bacterial endotoxin like LPS or endogenous pro-inflammatory cytokines (Yu et al., 2006). TLR4 binding of HMGB1 results in NF-kB upregulation (Park et al., 2006; Park et al., 2004), and it is thought that HMGB1 binding to TLR4 also leads to cytokine

release. For example, alcohol-induced HMGB1/TLR4 signaling is associated with induction of the inflammatory cytokine interleukin-1β (IL-1β) in the brain (Crews, Qin, Sheedy, Vetreno, & Zou, 2013). IL-1β is key to the inflammatory signaling cascade, and is considered the "gatekeeper of inflammation," given that it produces additional pro-inflammatory cytokines, induces the "sickness response," and is a critical mediator of peripheral-to-brain immune signaling (Dinarello, 2011; Maier & Watkins, 1998). In summary, TLR4 is implicated as a critical mediator of central and peripheral immune signaling cascades, and is activated through binding of LPS or HMGB1, which activates critical IL-1β-mediated signaling pathways.

An important characteristic of TLR4 is its location in the CNS and periphery. TLR4 is usually expressed in macrophages, and in the brain TLR4 is expressed in glial cells, particularly microglia (Lehnardt et al., 2003) which serve a primary role in neuroimmune defense. In healthy individuals, microglia are generally at rest, but can be transiently activated to respond to injury, insult or disease (Boche, Perry, & Nicoll, 2013). Activation of microglia through TLR4 signaling cascades results in the production of pro-inflammatory cytokines in the brain, and induces additional neuromodulatory mediators of inflammation, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Fernandez-Lizarbe, Pascual, & Guerri, 2009).

In the normal immune response, these and other downstream mediators serve a regulatory function that creates the inflammatory environment necessary to control infections or respond to injury. However, uncontrolled activation of the inflammatory response (as may be the case in heavy or chronic alcohol exposure) is associated with increased neuroinflammation, reduced neuroprotection and neuronal repair, and increased neurodegeneration (Guerri & Pascual, 2013). Next, we will discuss the specific impact of alcohol on inflammatory signaling, including possible consequences for neurocognition and frontal brain regions.

Alcohol and Inflammation

Recent evidence from human and animal research has converged to support the role of alcohol in promoting deleterious adaptations in inflammatory signaling cascades. In fact, it is likely that perturbation of the immune system is a critical mechanism in the etiology of AUD (Alfonso-Loeches & Guerri, 2011; Coller & Hutchinson, 2012; Leclercq et al., 2012; Leclercq, De Saeger, Delzenne, de Timary, & Stärkel, 2014; Mayfield et al., 2013), as alcohol appears to induce numerous pro-inflammatory effects throughout the CNS and periphery. Note that alcohol itself is not considered a true "danger signal," given that it is not a pathogen-derived molecule (Gallucci & Matzinger, 2001). Rather, alcohol is directly associated with the release of endogenous danger signals (e.g., LPS, HMGB1) within both the CNS and periphery. Peripherally, alcohol impairs the function of the intestinal barrier (Parlesak, Schäfer, Schütz, Bode, & Bode, 2000), and increases translocation of LPS from the gut (Wang, Zakhari, & Jung, 2010), thereby causing LPS levels in the blood to increase, as demonstrated by several studies (Bode, Kugler & Bode, 1987; Fujimoto et al., 2000; Leclercq et al., 2012; Parlesak, Schafer, Schutz, Bode & Bode, 2000). In general, higher circulating LPS in the blood indicates greater gut permeability, and is associated with inflammation in the context of alcohol use and alcoholic liver injury (Szabo et al., 2010). In the CNS, alcohol is also associated with the release of DAMPs such as HGMB1, which trigger neuroinflammatory cascades through activation of TLR4 (Szabo & Lippai, 2014). Perhaps more importantly, alcohol itself can directly bind to TLR4 in the brain, thereby stimulating neuroimmune signaling (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Lewis et al., 2013). Activation of the TLR4-mediated pathway by alcohol is associated with prolonged microglial activity and the aberrant

production of inflammatory mediators (e.g., pro-inflammatory cytokines, iNOS, COX-2) (Fernandez-Lizarbe et al., 2009).

In addition to inducing higher circulating levels of inflammatory markers, prolonged alcohol use is thought to be associated with greater sensitivity of cells to stimulation by LPS (Schäfer, Schips, Landig, Bode, & Bode, 1995). Indeed, a recent study collected whole blood cells following an acute binge-alcohol administration in humans and found an increase in circulating endotoxin in the blood, as well as increased inflammatory cytokines following an in vitro LPS stimulation procedure (Bala, Marcos, Gattu, Catalano, & Szabo, 2014). This suggests that even a single alcohol binge can acutely alter immune signaling, including responses to LPS.

Taken together, the evidence supports alcohol-induced dysregulation within both central and peripheral immune signaling pathways. One hypothesized consequence of such disrupted immune signaling is neuronal cell death in frontal control regions of the brain, consistent with the deficits in inhibitory control observed over the course of AUDs (Li, Luo, Yan, Bergquist, & Sinha, 2009). Examining the potential role of inflammation in mediating the relationship between alcohol use and cognitive impairment may be one avenue for exploring this hypothesis.

Alcohol, Inflammation and Neurocognition

The association between alcohol and neurocognitive deficits has been a topic of biomedical and psychological investigation for over 30 years (e.g., Bartholow, Henry, Lust, Saults, & Wood, 2012; Brandt, Butters, Ryan, & Bayog, 1983; Parsons & Leber, 1982; Trantham-Davidson et al., 2014). The suggestion that inflammatory processes may be involved in mediating this relationship is somewhat more recent (e.g., Bajaj et al., 2012; Butterworth, 1995). The mechanisms of alcohol-induced cellular damage are still not completely understood,

but likely involve oxidative stress associated with prolonged inflammation and microglial activation.

Briefly, increases in reactive oxygen species (ROS) occur as part of the TLR4-mediated inflammatory response (Crews & Nixon, 2009; Hensley, Robinson, Gabbita, Salsman, & Floyd, 2000). ROS induction is important for defending against pathogens, however high levels of ROS (i.e., during prolonged exposures to toxins or stressors, perhaps including alcohol) may result in significant cellular damage (Simon, Haj-Yehia, & Levi-Schaffer, 2000). Importantly, frontal brain regions may be especially vulnerable to damage caused by oxidative stress (Bartzokis, 2004). Recent animal and human studies have shed light on inflammation as a possible mediator of alcohol-induced frontal brain damage. One human post-mortem study found that earlier age of drinking onset is associated with increased expression of HMGB1 and TLR4 in the OFC (Vetreno, Qin, & Crews, 2013). In addition, a recent rodent study found that intermittent ethanol treatment in adolescent mice was associated with learning deficits and increased perseverative behavior in adulthood, as well as increased frontal cortical TLR4, TLR3 and HMGB1 (Vetreno & Crews, 2012). Further, binge-like ethanol administration in adolescent rats increased TLR4 gene expression in the PFC, and increased pro-inflammatory cytokines, including IL-1β (Pascual, Pla, Miñarro, & Guerri, 2014). Interestingly, this study also linked upregulation of TLR4 and other inflammatory mediators with alterations in myelin protein levels in the PFC. Such myelin changes are important because they may underlie long-term cognitive impairment. Note that the ethanol treatment in this study did not significantly alter myelin or inflammation in the brains of adult rats, suggesting that the age of onset of drinking should be accounted for when examining the impact of alcohol on neuroinflammation and cognition.

Conversely, suppression of neuroinflammatory signaling in rats has been found to prevent cognitive deficits (e.g., object memory recognition, conditioned taste aversion) associated with alcohol use (Tiwari & Chopra, 2013; Tiwari, Kuhad, & Chopra, 2009). Relatedly, adult mice lacking TLR4 receptors were protected from ethanol-induced inflammatory damage as well as the associated deficiencies in performance on cognitive tasks such as object memory recognition (Pascual, Baliño, Alfonso-Loeches, Aragón, & Guerri, 2011). Finally, the anti-inflammatory mediator IL-10 was negatively correlated with performance on a selective attention task in alcohol dependent subjects following 3 weeks of abstinence, highlighting the association between inflammation and cognitive impairments that may persist even into abstinence (Leclercq et al., 2012).

Taken together, these findings support a potential mechanistic link between chronic alcohol exposure, neuroinflammation, and cognitive impairments. We suggest that alcohol-induced alterations in inflammatory signaling could incite damage to frontal brain regions, thereby producing neurocognitive impairment and promoting the inhibitory control dysfunction characteristic of AUDs.

Alcohol and Epigenetics

Another likely relevant factor is epigenetics, which refers to the potentially reversible biochemical processes that regulate gene transcription and expression without altering the DNA sequence. Recent studies in animals and humans have suggested that epigenetic mechanisms may mediate the effect of alcohol on long lasting adaptations in the brain (Nestler, 2014; Starkman, Sakharkar, & Pandey, 2012). Epigenetic research has focused primarily on how changes to DNA influence chromatin structure (Warnault & Ron, 2013). DNA methylation is

one epigenetic process that has been shown to modify chromatin and exert downstream effects on gene transcription and expression of protein products. DNA methylation tends to occur at unmethylated cytosine guanine (CpG) dinucleotides, clusters of which are often located in promoter, or 5' regions, of many human genes, and have come to be known as CpG islands (Goldberg, Allis, & Bernstein, 2007). Unusual patterns of DNA methylation in humans have been associated with numerous diseases (Jones & Takai, 2001), including cancer, schizophrenia, and a variety of other psychiatric conditions including AUD (Santos, Mazzola, & Carvalho, 2005). Usually, higher ("hyper") methylation (in contrast to lower, "hypo" methylation) is associated with disease states, but this is can vary depending on the genomic location of the CpG site (Santos et al., 2005). Alcohol seems to be associated with modifications of gene expression due to both hypo- and hyper-methylation (Shukla et al., 2008).

Although still a somewhat nascent area of research, there are a growing number of studies on epigenetics and alcohol abuse in humans that have focused on DNA methylation. These findings support the hypothesis that alcohol exposure leads to aberrant changes in gene expression, which may have downstream effects on the development, progression and effects of AUDs (Wong, Mill, & Fernandes, 2011). Given the critical role of inflammatory signaling in AUD, methylation of genes that regulate TLR4 and influence the TLR4-mediated signaling cascade may be of particular interest from a pharmacological treatment perspective (e.g., Hagerty et al., 2016).

Epigenetics and Inflammation

Chronic inflammation is thought to be critically involved in promoting abnormal DNA methylation (Hur et al., 2011), and it has recently been suggested that epigenetics may serve as a

"unifying molecular mechanism to explain complex immune-mediated diseases" (Jenke & Zilbauer, 2012). Emerging evidence suggests this may be particularly true regarding AUD and alcohol-related illnesses, given that alcohol-induced epigenetic changes have been shown to impact several immune pathways associated with inflammatory responses (Curtis, Zahs, & Kovacs, 2013). Notably, LPS-induced TLR4 stimulation has been associated with epigenetic changes within regions of DNA that encode pro-inflammatory cytokines (Foster, Hargreaves, & Medzhitov, 2007). In addition, TLR4-dependent reprogramming of inflammatory genes appears to be mediated in part by epigenetic modifications (El Gazzar & McCall, 2010).

Relatedly, DNA methylation was shown to regulate transcription of IL-2, which serves a critical role in the immune response (Wen, Schaller, Dou, Hogaboam, & Kunkel, 2008). Further, alcohol-related epigenetic regulation of the inflammatory mediator IL-10 is indicated by the fact that decreased production of IL-10 appears to be partly responsible for increasing the sensitivity of chronically alcohol-exposed cells to LPS (Schäfer et al., 1995). To summarize, emerging literature suggests that epigenetic regulation may be involved in the immune response in the context of AUD. It is hypothesized that, that given the importance of TLR4 signaling in alcohol-related inflammatory signaling, examining DNA methylation or other epigenetic changes within the TLR4 gene may be an important avenue for future discovery.

TLR4 Methylation and Gene Expression

The association between DNA methylation levels and downstream effects (i.e., gene expression) is highly nuanced and can differ significantly across genes and regions, however methylation is frequently associated with decreased transcription of gene products (Jones, 2012). There is limited existing research that directly examines the relationship between methylation

and expression within the TLR4 gene, however several rodent studies support a link between TLR4 methylation and expression. Specifically, enhanced TLR4 expression was accompanied by TLR4 promotor demethylation in the context of a systemic inflammatory response (Chang et al., 2015). Another study found that suppression of TLR4 expression in mice may be mediated, at least in part, by TLR4 methylation, thereby reducing TLR4 responsiveness to an immune challenge (Zampetaki, Xiao, Zeng, Hu, & Xu, 2006). In addition to decreased immune responsivity, mice lacking in TLR4 receptors were also found to be protected against alcohol-induced changes within protein-degradation pathways, pointing to an important role for TLR4 in alcohol-related neurodegeneration (Pla, Pascual, Renau-Piqueras & Guerri, 2014). The role of TLR4 methylation/expression has not been explicitly addressed in human studies to date, however our group recently demonstrated that TLR4 methylation may be protective against the damage conferred by alcohol on gray matter within two specific brain regions, the inferior parietal cortex and the precuneus (Karoly et al., in press). Specifically, we found a significant negative association between alcohol dependence severity and precuneus and inferior parietal gray matter in individuals with low TLR4 methylation, but no relationship between alcohol and gray matter in individuals with high levels of TLR4 methylation. These results support the idea that that TLR4 methylation may downregulate inflammatory signaling in the brain, and perhaps serve a protective role against the damage conferred by alcohol on the brain.

The Present Study

Although there has been substantial research on reward and control networks and how they may be related to AUD treatment outcomes, considerably less work has focused on delineating the molecular mechanisms that underlie deleterious alcohol-related neuroadaptations within

these brain networks. Examining molecular precursors of these adaptations may aid in ultimately developing new pharmacological treatment targets.

The existing literature suggests that central and peripheral inflammation likely plays an important role in the etiology of alcohol use disorders via TLR4-mediated signaling cascades (Alfonso-Loeches & Guerri, 2011; Fernandez-Lizarbe et al., 2009; Mayfield et al., 2013). In addition, TLR4 methylation may be a key regulatory mechanism that impacts inflammatory signaling (Takahashi, Sugi, Hosono, & Kaminogawa, 2009), and recent data from our laboratory indicates that TLR4 is differentially methylated in alcohol use disorders (Hagerty et al., 2016). Taken together, this prior evidence suggests that alcohol-related perturbations of gene expression within the TLR4-mediated inflammatory signaling cascade may impact cognitive function, alter neural control circuitry and perhaps contribute to impaired control over drinking and relapse (Bartzokis, 2004; Leclercq et al., 2012; Tiwari & Chopra, 2013; Vetreno et al., 2013). Thus, the goal of the present study was to examine the complex relationship between inflammation, TLR4 methylation and cognitive functioning among heavy and light drinkers. Figure 1 depicts the hypothesized inflammatory pathway through which alcohol may act on the brain and periphery to ultimately damage frontal control regions of the brain.

Importantly, one of the primary limitations of the prior literature is the inability to measure inflammation in the brain in vivo. Because alcohol is thought to cause neuroinflammation, and this neuroinflammation may be partially due to peripheral inflammation, we sampled peripheral inflammation in blood as a surrogate marker for neuroinflammation. We selected IL-1β, IL8, IL-6 and HMGB1 as the specific inflammatory cytokines to examine based on our preliminary findings (see below) as well as support from the literature (e.g., Crews et al., 2013; Leclercq et

al., 2014a; Pascual et al., 2014). We measured basal levels of cytokine gene expression, as well as cytokine expression following an LPS stimulation procedure (Bala et al 2014).

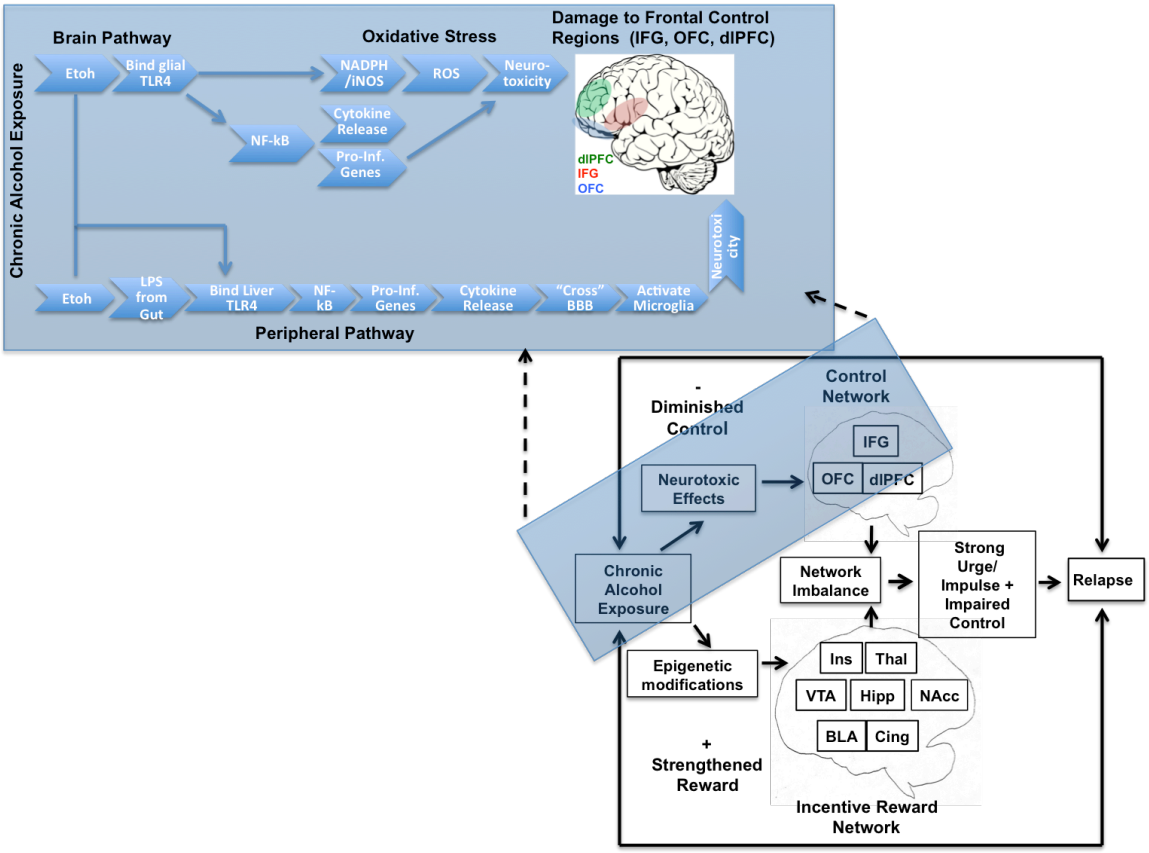

Figure 1. Theoretical model incorporating the role of neuroinflammation within the neural control pathway**.** This model illustrates the hypothesized impact of chronic alcohol exposure on reward and control networks in the brain. Regarding control systems, the model shows that chronic alcohol exposure leads to neurotoxic effects which influence inhibitory control regions of the brain, including the inferior frontal gyrus (IFC), orbitofrontal gyrus (OFC) and dorsolateral prefrontal cortex (dlPFC), and these changes are associated with an overall decrease in the connectivity strength of the control network in the brain. The imbalance between the strength of the reward and control network is such that the urge to use substances (reward network) eventually overpowers the ability to control substance use (control network), and this ultimately leads to relapse, thereby perpetuating the cycle of chronic alcohol use. The top left portion of the figure shows in greater depth the molecular mechanisms through which alcohol may exert neurotoxic effects and ultimately damage frontal control regions of the brain. As shown in the large blue box, alcohol is related to neuroinflammation directly, and similarly exerts peripheral effects. Chronic alcohol poses a significant challenge to the immune system, and peripherally, it impairs the intestinal barrier and allows the release of endotoxins and other danger signals, including LPS, which binds to toll-like receptor 4 (TLR4) in the liver. TLR4 triggers signaling pathways, via Nf-kB, that lead to transcription of pro-inflammatory genes and the release of pro-inflammatory mediators, (e.g., cytokines)

into the blood. The mechanism through which peripheral cytokines influence the brain is still not completely understood, but evidence suggests that some cytokine molecules may be able to permeate the blood brain barrier (BBB) either directly (i.e., active transport) or indirectly. In the brain, prolonged microglial activation is associated with neurodegeneration, and may lead to impairments in frontal brain regions including the dlPFC, OFC and IFG. Alcohol also exerts more direct effects on the brain, through directly binding to glial TLR4 receptors, which initiates the same signaling cascade (i.e., via Nf-kB), resulting in an increase of pro-inflammatory cytokines circulating in the brain. These substances can have damaging effects on neural cells. In particular, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and inducible nitric oxide (iNOS) are increased, which leads to reactive oxygen species (ROS) production and subsequent neurotoxicity due to oxidative stress.

Study Aims and Hypotheses

The study was designed to measure the relationships denoted by each path in the model in Figure 2. For the relationships between alcohol and the inflammatory measures, and between alcohol and the cognitive measures, alcohol was examined both as a grouping variable and as a continuous predictor. For significant regression analyses, we conducted follow-up moderation models exploring the potential moderating role of any significant covariates besides alcohol use. If the relationships indicated by either 1) paths A and B, and/or 2) path C (see Figure 2), are significant, we planned to conduct a mediation test, examining whether any of the inflammation variables mediate the relationship between alcohol and cognition. Finally, as an exploratory aim, we examined the correlations between TLR4 methylation and circulating and stimulated cytokines. Specific hypotheses are outlined in detail below.

Aim 1. The first aim of the study was to examine path A (Fig 2). Specifically, we hypothesized that heavy drinkers compared to light drinkers would show greater levels of circulating and LPS-stimulated cytokines (e.g., IL-1β, IL8, IL-6 and HMGB1), as well as greater TLR4 methylation. Further, we hypothesized that alcohol consumption measured continuously would be positively associated with circulating and stimulated cytokines, as well as TLR4 methylation. If any covariates were also known to have theoretical associations with alcohol use

and inflammation), then follow-up tests of moderation were conducted. Importantly, we performed cell counting to assay the number of monocytes present in each sample and thereby ensure that any potential differences in cytokines that we observed between heavy and light drinkers could not be attributed to differences in monocytes. If the heavy and light drinkers demonstrated different monocyte counts, it would be necessary to follow-up with flow cytometry to further characterize the difference in cell populations.

Aim 2. The second aim was to examine path B (Fig. 2). Specifically, we hypothesized that inflammatory markers (i.e., circulating and stimulated cytokines, as well as TLR4 methylation) would positively predict performance on cognitive tests.

Aim 3. For the third aim, we examined path C (Fig.2). We tested the hypothesis that heavy drinkers would show lower performance on a battery of cognitive measures compared to light drinkers, and that alcohol consumption measured continuously would be a significant negative predictor of cognitive performance. We further hypothesized that the relationship between alcohol use and scores on these cognitive measures would be mediated by cytokines.

Exploratory Aim. As an exploratory aim, we tested the hypotheses that methylation within the TLR4 gene would be associated with basal cytokine gene expression (e.g., IL-1β, IL8, IL-6 and HMGB1), as well as with cytokine expression after an LPS stimulation procedure. Given that methylation is often associated with decreased expression of the protein product (Newell-Price, Clark, & King, 2000), we hypothesized that greater TLR4 methylation would be associated with decreased circulating and stimulated cytokines.

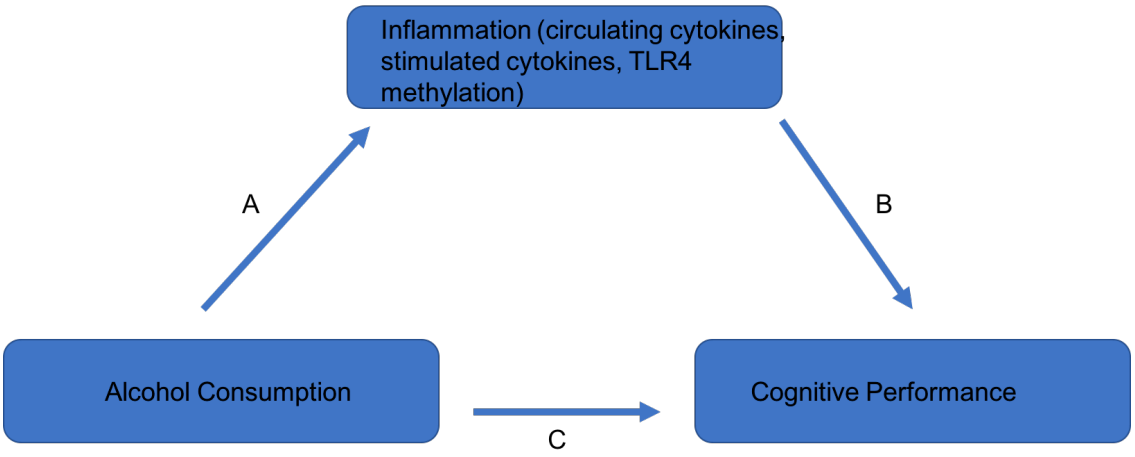

Figure 2. Model depicting the 3 relationships hypothesized in primary study aims.

Preliminary Studies

Alcohol and inflammation. We recently examined the association between pro-inflammatory cytokines in blood and alcohol use phenotypes. Although our sample size was small and the blood samples analyzed were several years old, we still observed a number of significant correlations between clinical alcohol phenotypes and plasma inflammatory markers. In particular, we found that interleukin-8 (IL-8) was significantly correlated with failed control over drinking (r=.543, p=.002). We also found moderate correlations between neural connectivity within executive control brain regions and plasma IL-8 levels (r = -.24 to r=-.55) (Hutchison et al., unpublished data). To follow up on these preliminary findings, we measured expression of IL-8 in the present study.

Alcohol, TLR4 and Brain Volume. We examined whether TLR4 methylation moderates the relationship between alcohol use and gray matter (GM) within particular regions of the brain that were previously identified as being negatively associated with alcohol use (Thayer et al., 2016). In these analyses, we examined TLR4 methylation and GM thickness in a large sample (N=707; 441 male) of adults (ages 18-56) reporting a range of AUD severity (mean AUDIT

score=13.18; *SD*=8.02). We ran a series of Ordinary Least Squares (OLS) regression models to regress GM separately in four bilateral brain regions (precuneus, lateral orbitofrontal, inferior parietal and superior temporal) on alcohol use variables, TLR4 methylation, and the Alcohol by TLR4 methylation interaction, controlling for demographic, psychological and other substance use variables. We observed a significant Alcohol by TLR4 methylation interaction in the equations modeling left precuneus and right inferior parietal GM as the dependent variables. Follow-up analyses examining the nature of these interactions indicated the presence of a significant negative association between alcohol and precuneus and inferior parietal GM in individuals with low TLR4 methylation, but no relationship between alcohol and GM in the high methylation group. Although preliminary, these findings suggest that TLR4 methylation may be protective against the damage conferred by alcohol on precuneus and inferior parietal GM, thereby implicating TLR4 for further investigation as a possible AUD treatment target (Karoly et al., in press).

TLR4 Methylation and Subjective Responses to Alcohol. In a similar study, we sought to elucidate the relationship between TLR4 methylation and subjective responses to alcohol, as this could potentially shed light on the role of TLR4 in promoting AUDs, and thereby highlight the potential of TLR4 modulation as a treatment option. We used latent growth models to examine the relationship between TLR4 methylation (collected from saliva samples) and subjective responses during an intravenous alcohol infusion session across 221 heavy and light-to-moderate drinkers. Across the entire sample, TLR4 methylation was associated with greater baseline happiness, and a greater increase in happiness over the course of the infusion. Among heavy drinkers, TLR4 methylation was associated with lower levels of baseline stimulation and tension, but greater increases in stimulation during the infusion. These findings indicate that

TLR4 methylation is associated with not only differences in baseline affect, but also changes in subjective arousal and affective states during acute alcohol intoxication, which differ based on self-reported levels of alcohol use severity. Overall, these data support TLR4's potentially important role in influencing subjective responses to alcohol, and indicate a need for further research on its potential as a pharmacological treatment target (Karoly et al., under review).

CHAPTER II

METHOD

Study Design

The study is a non-equivalent control group cross-sectional design, including two self-selected groups (heavy drinkers vs. light drinkers). The study involves a single experimental session in which participants provided a blood sample, completed a battery of assessments of psychological and substance use factors and a battery of cognitive assessments. A detailed outline of all study assessments is provided below.

Power Analysis

Using G*Power 3.1 (Faul, Erdfelder, Lang, & Buchner, 2007), sample size calculations were computed for a 2-group comparison, using a one-tailed alpha level of .05 (given our directional hypotheses that the heavy drinking group will exhibit greater inflammation), at a power level of .80 (Cohen, 1988). Sample size was selected to permit analysis of hypotheses from the primary aim (aim 1) regarding comparison of heavy and light drinkers on expression of inflammatory cytokines.

Our preliminary data analysis examining the relationship between blood cytokines and alcohol dependence found correlations between r=.2 to r=.5, which corresponds with a medium to large effect size (Cohen, 2013). Similar effect sizes were found in our prior analyses of DNA methylation among heavy drinkers. Based on this work, we anticipated the magnitude of the effect comparing inflammatory markers between the two groups in the present study to be moderate (i.e., Cohen's *d*=.5) to large (i.e., Cohen's *d*=.8)

Anticipating a moderate effect size for a between factors, two group comparison over a single time point, the study requires 102 participants (51 in each group) to test the first hypothesis. However, anticipating the magnitude of the effect to be large (Cohen's *d*=.8), 21 participants are needed in each group (n=42 total). Assuming the magnitude of the effect to be between medium and large (Cohen's *d*=.65), a total of 60 participants are needed, with 30 per group. Thus, to be somewhat more conservative, we planned to recruit a total of 80 participants (40 heavy drinkers and 40 light drinkers).

Similarly, anticipating a moderate effect size (f^2=.15) for a linear multiple regression, fixed model, with an R-squared deviation from zero, including 3 predictors (e.g., alcohol use and 2 covariates), the proposed study requires a total of 77 participants. Thus, we planned to obtain approximately 80 subjects total. Note that we are powering the main effect, rather than attempting to power an interaction in the case of a moderation model, given the difficulty of adequately powering an interaction term (McClelland & Judd, 1993).

Subject Selection

A total of 82 male and female participants between the ages of 25-40 were recruited from the greater Boulder-Denver area. We recruited 43 heavy drinkers, defined as drinking 5 or more drinks (4 or more drinks for women) on the same occasion on at least 5 days per month. We also recruited 39 light drinkers, defined as consuming no more than two alcoholic drinks per occasion, and drinking on no more than two days per week. Age range was selected to minimize the impact of development and aging on inflammatory markers and cognitive function (Barrientos, Frank, Watkins, & Maier, 2010; Jaspan, Lawn, Safrit, & Bekker, 2006).

Regarding eligibility for this study, we applied exclusionary criteria based on having or being treated for serious medical or mental illness (e.g., cancer, psychotic spectrum disorders),

reporting recent substance/medication use and pregnancy. Specific inclusion criteria for the present study were: (1) Age(s) 25-40; (2) Non-every day smoker and/or tobacco user; (i.e. smoking less than 4 days out of the week; (3) No serious medical illness or injury within past 6 months; (4) Not on psychotropic medications and not currently under treatment for any psychiatric and/or neurological disorder; (5) Never received a diagnosis of bipolar disorder or a diagnosis of psychotic spectrum disorder; (6) Never received a diagnosis of a neurological disorder (e.g. epilepsy); (7) No use of cocaine, methamphetamine, heroin or other illicit drugs (except marijuana) in the previous 60 days (assessed on the phone screen and TLFB interview); (8) Never received treatment of any kind for alcohol abuse; (9) Being either a heavy drinker, defined as drinking 5 or more drinks (4 or more drinks for women) on the same occasion on at least 5 days in the 30 days prior to beginning the study, or a light drinker, defined as consuming no more than two alcoholic drinks per occasion, and drinking on no more than two days per week during the 30 days prior to beginning the study. Subjects were also asked on the phone screen whether their drinking patterns had been relatively consistent over the past year, and individuals for whom this was not the case were screened out (e.g., if a subject had been a heavy drinker previously and only recently cut down to light drinking).

Recruitment and Eligibility Screening. Participants were recruited from the greater Boulder County area through flyers and advertisements placed around the community and in local newspapers and online web forums such as craigslist. Recruitment materials described the opportunity to participate in a study about alcohol use. The advertisements also indicated the opportunity to earn $50 in cash for participation. The advertisements instructed interested individuals to call a toll-free number and indicate their interest in participating in the study.

Individuals who indicated interest in participating in the study were then taken through an initial phone-screening session to determine eligibility. In the pre-screening process, a study research coordinator or research assistant read an IRB approved phone screening script to all interested participants. These screening materials were used to determine whether the interested individual was eligible to participant in the study, according to the specific exclusion/inclusion criteria outlined above. Prior to completing the phone-screen, participants were informed that they would be asked questions regarding substance use behavior, and it was ensured that the participants were in an environment in which they were comfortable answering such questions. They were given the option of rescheduling the phone screening session if this was not the case. Participants were also informed that they should not answer any question that makes them uncomfortable, but that failing to answer some questions may result in being ineligible to participate in the study. Following completion of the screening questions, the study personnel conducting the interview read information about the study to the participant, and gave them an opportunity to ask questions about their potential participation in the research. All individuals deemed eligible by the phone screen were informed of the study procedures and assessments, including the self-report measures, cognitive assessments and blood collection. They were also informed of any potential risks involved with participating in the study. All eligible individuals who were interested in participating were scheduled for their appointment upon verbal indication of their willingness to participate in the study. These individuals were also sent an e-mail that included the time and date of their appointment as well as directions informing them how to get to the Center for Innovation and Creativity (CINC), where the study takes place.

Study Procedures

Once qualified participants arrived at the CINC, they (1) completed an informed consent form; (2) completed a breath alcohol test; (3) provided a blood sample for assays of peripheral inflammatory markers; (4) completed computerized and researcher-administered questionnaires and assessments and (5) completed a cognitive testing battery.

Upon arrival at the CINC, participants were met by a member of the study team (i.e., a research assistant or graduate student) who escorted them to a private consultation room. Here, participants were asked to complete the informed consent form. Prior to asking the participant to sign the form, the trained member of the study team and the participant had a discussion regarding the research study. Additionally, the research assistant or graduate student was available to answer any questions the participant had about the study. Participation was clearly stated as voluntary, with the option to withdraw from the study at any time. There was no deception involved with any aspect of this study. After discussing the study and going over the consent form with the researcher, the participant signed the informed consent document.

To ensure that participants were not currently under the influence of any substances while completing the study, they were asked ahead of time to refrain from drinking alcohol within 48 hours, smoking marijuana within 6 hours or smoking cigarettes within 2 hours of the session. At the start of this session, all participants were breathalyzed to ensure that they had no alcohol in their systems. Only participants with a breath alcohol level (BrAC) of zero were allowed to participate in the study. Any participant who blew a BrAC over 0.00 was given one chance to reschedule his or her appointment for the next day. If s/he came in the next day with a breath alcohol greater than 0.00 then this session was counted as “missed.” If a subject accumulated a “missed” study session, they were automatically dropped from the study on the grounds that they

failed to follow explicit study directions properly. Only one subject was dropped from the study due to blowing a BrAC over 0.00. Participants received no payment if a session was terminated due to failure to follow the abstinence instructions; they were informed of this contingency during the phone screening when their session was scheduled.

Following a successful pass of the BrAC screen, participants were asked to provide a small sample of blood for the inflammatory markers analysis. This allowed us to examine the relationship between peripheral inflammation, epigenetics and alcohol use. It is important to note that a blood sample was necessary to measure these markers (e.g. cytokines). The blood samples were collected by a member of our research lab who is certified in phlebotomy. The blood draw procedures involved collecting venous blood (up to 63 mL, or 4.26 tablespoons) through venipuncture of a peripheral arm vein using standard, sterile phlebotomy techniques. The samples were tested for inflammatory cytokine levels using equipment in our lab facility at the CINC.

Following the blood draw, participants were asked to fill out computerized baseline questionnaires, and a trained research assistant administered a Time Line Follow Back (TLFB) interview to the participant. Next, the participant completed a battery of cognitive assessments. Participants received payment upon completing the blood draw and cognitive battery and questionnaires.

Assessments and Measures

Self-Report Measures

The measures listed below are drawn from a standard assessment battery administered to alcohol dependent subjects in our laboratory. Several of these measures are specifically intended to generate scores that could be used as covariates in planned statistical analyses (e.g., sleep,

stress, aerobic exercise and fruit and vegetable consumption measures), given that these instruments all measure variables known to impact inflammation, epigenetics and/or cognitive performance. However, we also included several standard measures of alcohol use and psychological functioning to allow for some flexibility in the potential follow-up analyses that could be conducted using these data in the future. In regression analyses, covariates were included if they demonstrated at least a theoretical association with the dependent variable in each model (i.e., inflammation, epigenetics or cognitive performance), and demonstrated significant relationships with the independent variables in a given model within the present sample (as indicated in Tables 2-4). For each self-report measure discussed below, potential associations with inflammation, epigenetics and/or cognitive performance have been indicated.

Demographics. A demographics questionnaire was used to collect information on age, sex, marital status, SES, occupation, income, education, and race. Age has been shown to impact both inflammation (Chung et al., 2009) and cognition (Salthouse, 2013), and gender differences in inflammation have been observed in the context of alcohol use (Pai et al., 2006).

Quantity/Frequency of Alcohol Use. Alcohol use was evaluated with a variation of the measure used by White and Labouvie (White & Labouvie, 1989). First, participants are asked if they have ever had an alcoholic drink (with instructions that define one alcoholic drink as "one beer, one glass of wine, or one serving of hard liquor either by itself or in a mixed drink"). All participants recruited for this study should answer yes to this question, given that alcohol use was part of the inclusion criteria. Participants are then asked to rate: (1) their frequency of use in the last three months on a 9-point scale ranging from "never" to "every day", (2) their typical quantity of drinks in one sitting on a 10-point scale ranging from "no drinks" to "more than 20

drinks", and (3) their frequency of getting drunk when drinking in the past three months on a 5-point scale ranging from "never" to "always."

Alcohol Use Disorders Identification Test (AUDIT). The AUDIT (Saunders, Aasland, Babor, de la Fuente, & Grant, 1993) was used to detect less severe problem drinkers and addresses both current problems (problems within the last 3 months) and problems across an individual's lifetime. The AUDIT consists of ten questions that cover such domains as alcohol consumption, drinking behavior, adverse psychological reactions, and alcohol-related problems ($\alpha = .855$).

Drinking History Questionnaire. Drinking History is measured with 9 items assessing lifetime drinking history such as current drinking, age of onset, and number of previous attempts to quit/reduce drinking. For the present study, we only examined the drinking onset item "How old were you when you started to drink regularly?"

Alcohol Dependence Scale (ADS). The ADS has been used widely and found to have excellent predictive value with respect to DSM diagnosis (Kivlahan, Sher, & Donovan, 1989) and was used to assess severity of alcohol use symptoms ($\alpha = .838$).

Beck Depression Inventory-II (BDI-II). The BDI-II (Beck, Steer, & Brown, 1996) is a 21-item measure of depression symptom severity in the past two weeks. BDI-II scores range between 0 and 63, with categorical depression ratings of "minimal" (0–13), "mild" (14–19), "moderate" (20–28), and "severe" (29–63) ($\alpha = .920$). Depression is associated with inflammation (Yirmiya, 2000) and cognitive performance (McDermott & Ebmeier, 2009). Thus, BDI scores were used as covariates when appropriate in these analyses.

Beck Anxiety Inventory (BAI). The BAI (Beck & Steer, 1991) consists of 21 items, each describing a common symptom of anxiety. The items are summed to obtain a total score that can range from 0 to 63 ($\alpha = .921$).

Fruit and Vegetable Screener (the "All-Day" Version). This 10-item measure was developed by the National Cancer Institute to assess how many times in the previous month subjects consumed different types of fruits and vegetables, including potion size questions for every food item. This measure was shown to be a useful estimate for obtaining median intakes of fruit and vegetable servings in U.S. populations (Thompson et al., 2002). Fruit and vegetable consumption has been shown to influence inflammation (e.g., Holt et al., 2009), and dietary factors have also been linked with epigenetic changes (see Hardy & Tollefsbol, 2011) and cognitive function (Lourida, et al., 2013). Thus, the inclusion of this measure allows diet to be controlled for in regression analyses ($\alpha = .768$). In all analyses, a total sum score of all daily fruits and vegetable servings was used.

Voluntary Aerobic Exercise Questionnaire (VAEQ). The VAEQ assesses levels of voluntary exercise (Bryan and Rocheleau, 2002). Participants indicate how frequently they engaged in exercise activities in the past 3 months and past week. The exercise composite score used in these analyses was composed of 4 items: (i) "In the past 3 months, what is the average number of days per week that you engaged in aerobic exercise?" (ii) "In the past 3 months, what is the average number of total minutes per week that you engaged in aerobic exercise?" (iii) "In the past week, how many days did you engage in aerobic exercise?" and (iv) "In the past week, what is the total number of minutes that you engaged in aerobic exercise?" This measure has been used to quantify exercise participation in our previous alcohol studies (Karoly et al., 2013). Given that exercise may impact inflammation (see Woods, Vieira, & Keylock, 2009) and epigenetics (see Bryan, Magnan, Hooper, Harlaar, & Hutchison, 2013; Ntanasis-Stathopoulos, Tzanninis, Philippou, & Koutsilieris, 2013), this measure allowed us to control for exercise behavior in inflammation and epigenetic analyses ($\alpha = .940$).

Pittsburgh Sleep Quality Index (PSQI). The PSQI (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989) is a 19-item self-report scale that measures sleep quality over the past month. The 19 self-related items are combined to form seven "component" scores, each of which has a range of 0-3 points. In all cases, a score of "0" indicates no difficulty, while a score of "3" indicates severe difficulty. The seven component scores are then added to yield one "global" score, with a range of 0-21 points. In the present study, we controlled for sleep duration, given that sleep is known to be associated with both inflammation (e.g., Patel et al., 2009) and epigenetic factors (see Qureshi & Mehler, 2014). Thus, the sleep duration subscale was used as a covariate in epigenetic and inflammation analyses ($\alpha = .609$).

Perceived Stress Scale (PSS). The PSS (Cohen, Kamarck, & Mermelstein, 1983) is a 14-item scale that measures the degree to which situations in someone's life are perceived as stressful. Stress is a known correlate of substance use and abuse. Participants' perceived stress levels may be associated with inflammatory processes (e.g., Hart & Kamm, 2002), epigenetic changes (see Nestler, 2012) and cognitive function (McEwen & Sapolsky, 1995). Thus, this measure was collected so that we could control for stress in regression analyses ($\alpha = .275$).

Timeline Follow Back (TLFB). The TLFB (Sobell, Sobell, & Maisto, 1979) is an assessment method that obtains estimates of daily alcohol, cigarette, and drug use. The TLFB has been shown to have good psychometric characteristics with a variety of drinker groups and can generate variables that provide a wide range of information about an individual's drinking (e.g., pattern, variability, and magnitude of drinking). This instrument requires subjects to recall from memory the number of drinks consumed for each day over the prior 90 days as well as their use of tobacco products and recreational drugs. Studies with alcoholic dependent individuals have shown this instrument to be reliable in assessing drinking frequencies and other behaviors such

as arrests and hospitalizations (Sobell et al., 1979). We used TLFB cannabis days as a covariate in regression analyses, given the established associations between cannabis use and inflammation (Keen, Pereira, & Latimer, 2014) and cognitive performance (Curran et al., 2016).

Cognitive Assessments.

To assess cognitive function, we administered a battery of tests that assess attention and working memory, two critical components of executive function. These tests were selected from the NIH Toolbox, which includes a variety of state of the art cognitive assessments with high reliability and validity. All NIH Toolbox measures have been normed and validated across the lifespan in participants ages 3-85. The NIH Toolbox validation studies were conducted across this entire age range, typically including 450-500 subjects. Further, validation involved statistically comparing results from the NIH Toolbox against existing "gold standard" measures, whenever available. For each test, the mean score is 100, with a standard deviation of 15. The cognitive battery included in the present study includes the following tests:

Flanker Inhibitory Control and Attention Task. The Flanker is a measure of the attention and inhibitory control components of executive function. Specifically, this test measures the allocation of one's limited capacities to deal with an abundance of environmental stimulation. The Flanker requires the participant to focus on one particular stimulus on the screen while inhibiting attention to the stimuli flanking it.

The List Sorting Working Memory Test. This task assesses working memory, which refers to the capacity of an individual to hold information in a short-term buffer and manipulate the information. The task requires the participant to recall and sequence different visual and auditory stimuli. Pictures of different foods and animals are displayed with both an accompanying audio recording and written text that names the item. The participant is asked to say the items back to

the examiner in size order from smallest to largest.

Overall, this battery covers cognitive domains that have been suggested to be related to inflammation and alcohol use (e.g., Ambrose, Bowden, & Whelan, 2001; Leclercq et al., 2012).

Blood Procedures

Collection of Blood Samples

Blood samples were collected by a member of our research team who has completed a certified training in phlebotomy. Up to 63 mL (4.26 tablespoons) of blood was collected from each subject to provide adequate samples of serum, plasma, and whole blood for all inflammation and epigenetic assays. In some cases, subjects were not able to provide the full 63 mL due to dehydration or discomfort with the blood draw.

LPS Stimulation of Whole Blood

Fresh whole blood (10 mL) was added to a 50 mL conical tube and diluted 1:1 with warm RPMI-1640 (Sigma-Aldrich) with 10% Fetal Bovine Serum (FBS) (Thermo Fisher). After gently mixing, 3 mL of blood was added to each well in a 6-well culture plate (Thermo Fisher). Three wells were stimulated with 25 ng/ml of LPS (Sigma-Aldrich) and the same volume of PBS that was used to dissolve LPS was added to the remaining three wells as a control. The culture plate was incubated for 24 hours at 37ºC, 5% CO2. After the 24-hour incubation period, the cell count was first measured from each condition (LPS and control) as described in these methods. Blood from each condition was gently mixed inside each well and transferred to a 15 mL conical tube and centrifuged at 400xg for 15 minutes to separate plasma. The upper plasma layer from each condition was aliquoted for each enzyme-linked immunosorbent assay (ELISA) and stored at -80^{o}C for analyses.

Determining Cell Population

The total PBMC and monocyte count were measured before and after LPS treatment. Immediately after blood collection, 200 µl of whole blood was resuspended in 2 ml of red blood cell (RBC) lysis buffer (Qiagen), gently mixed, and incubated for 10 minutes at room temperature. Cells were centrifuged (400xg, 5 min) to remove the lysis buffer. The cell pellet was resuspended in 200 µl of phosphate buffered saline (PBS) and incubated at room temperature for 10 minutes. A small volume of the resuspended cells (20 µl) was transferred into a 1.5 ml microcentrifuge tube and diluted 1:10 in PBS. Cells were counted using the Millipore Scepter 2.0 automated cell counter with a 40 µm sensor tip. The Millipore Scepter utilizes the Coulter principle to differentiate cells based on cell size (diameter). Cell counts and size distributions are displayed as a histogram on the Scepter monitor. Cell counts determined using this method are comparable to cell counts obtained with Flow Cytometry (Cappione, Crosslet, Thirumalapura & Hoover, 2011). Previously reported cell size ranges for PBMC (7-12 µm) and monocytes (9-12 µm) were used to determine their concentration present in the sample. The same procedure was performed on the LPS-treated and untreated cultured blood.

Isolation and Cryopreservation of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated by Histopaque-1077 density gradient centrifugation (Sigma) and cryopreserved in FBS with 10% DMSO to potentially perform flow cytometry in the future and to obtain a more precise cell count. In lieu of flow cytometry, isolated PBMC's were counted using the Millipore Scepter 2.0 cell counter.

Within one hour after blood collection, 10 ml of blood was diluted 1:1 with PBS and gently layered onto 10 ml of room temperature Histopaque-1077 (Sigma-Aldrich) in a 50 ml conical tube and centrifuged (500xg, 20 min) with no brake. Following centrifugation, the PBMC layer was carefully removed using a Pasteur pipette and transferred to a new 15 ml tube.

The PBMC fraction was washed using 6 ml of PBS and centrifuged (400xg, 10 min). Wash step was repeated once. The PBMC pellet was resuspended in 1 ml of PBS and 20 µl of the cell suspension was transferred to a 1.5 ml microcentrifuge tube and diluted 1:10 with PBS and the cell count was determined using the Millipore Scepter 2.0.

The remaining cell suspension was centrifuged (400xg, 10 min) and the cell pellet was resuspended in media consisting of FBS with 10% DMSO and stored at -80^{o}C. In order to obtain an optimal freezing rate for cell preservation, the cell sample was cooled at a rate of -1^{o}C per minute using a Mr. Frosty^{TM} freezing container (Thermo Scientific).

Circulating Cytokine Assays

Previous studies in animals and humans (Bala et al., 2014; Mayfield et al., 2013), as well as our own preliminary data, suggest that cytokine activation is associated with the pathophysiology of AUDs. Using our blood samples, circulating levels of IL-1β, IL-6, and IL-8 were measured in cultured whole blood.

Within 30 minutes of blood collection, plasma was separated from blood cells by centrifugation (1000xg, 15 minutes). Plasma was aliquoted and stored at -80^{o}C until immunoassays were performed. Circulating plasma levels of IL-1β, IL-6, and IL-8 were measured using a quantitative high-sensitivity sandwich enzyme immunoassay technique (Quantikine HS ELISA, R&D Systems), following the manufacturer's instructions. Prior to performing the assay, samples and reagents were brought to room temperature (18-25°C) without additional heating. Plasma IL-1β concentration was measured using IL-1 beta/IL-1F2 Quantikine HS ELISA, IL-6 using Human IL-6 Quantikine HS ELISA, and IL-8 using Human CXCL8/IL-8 Quantikine HS ELISA. Plasma samples were undiluted, ran in duplicate, and measured using a

microplate reader Elx800 (Biotek Inc, Winooski, VT) set to the wavelength suggested by the manufacturer.

LPS-Stimulated Whole Blood Immunoassays

IL-1β, IL-6, and IL-8 concentrations in LPS-stimulated blood were quantified using DuoSet ELISA Development System kits (R&D Systems) following the manufacturer's instructions. As previously described above, blood was collected in EDTA tubes and centrifuged (1000×g, 15 min) within 30 minutes of collection to separate plasma. Plasma was aliquoted and stored at -80^{o}C. Prior to performing the assay, samples and reagents were brought to room temperature (18-25°C) without additional heating. Plasma was diluted 1:10 in reagent diluent to measure IL-1β, diluted 1:20 to measure IL-6, and 1:5 to measure IL-8. The absorbance was read using a microplate reader Elx800 at 450 nm.

HMGB1 Production

Circulating HMGB1 concentration in plasma, as well as LPS-stimulated HMGB1 in plasma, were quantitated by Human HMG1/HMGB1 sandwich ELISA assay (LifeSpan Biosciences) according to the manufacturer's instructions. For circulating assays, plasma was diluted 1:400 in sample diluent and measured using a microplate reader Elx800 at 450 nm. For LPS-stimulated assays, plasma was diluted 1:100 in sample diluent.

DNA Extraction and Storage

Genomic DNA was isolated from 10 ml of blood collected in EDTA tubes using Gentra Purgene Blood Kit (Gentra, Minneapolis, MN) according to the manufacturer's protocol. Isolated DNA was stored in 1 ml of DNA hydration solution in cryovials at -80^{o}C

Bisulfite Sequencing / Estimation of Methylation

Blood samples were assayed to measure methylation of certain CpG sites within the inflammatory gene TLR4. Methylation of CpG sites within this gene has been previously demonstrated to be associated with alcohol dependence (Hagerty et al., 2016).

To determine the methylation of CpG sites near the TLR4 promotor, pyrosequencing was performed at EpigenDX (Worcester, MA). Pyrosequencing quantitatively monitors the real-time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a proportional light signal (Tost & Gut, 2007). The assay covered 4 CG dinucleotides in the first exon after the 5' untranslated transcription start site, ranging from +27 to +54 in reference to the translational start site (Figure 3). Site analysis was based on the ability to generate primers located around CpG islands and that meet the requirements for accurate pyrosequencing. All primers are owned by EpigenDx.

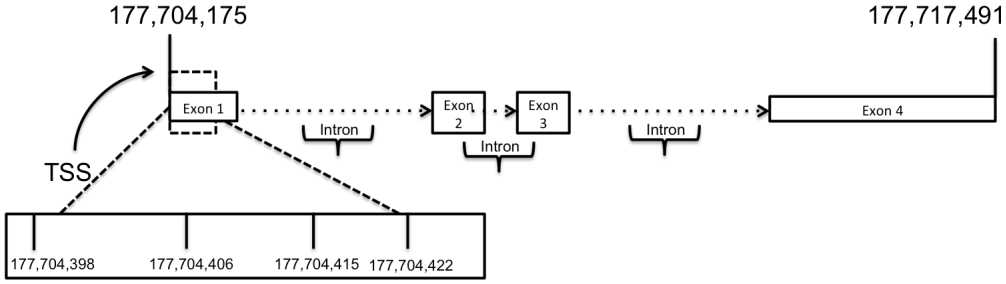

Figure 3. Assayed CpG sites fell within the first exon of the TLR4 gene. Selected CpG sites were chosen on the basis of their close proximity to the transcription start site (TSS) and location within an important regulatory region of the gene.

Bisulfite treatment by EpigenDx was performed on 500 ng of genomic DNA using a proprietary bisulfite salt solution. DNA is diluted to 45 µL and 5 µL of 3N NaOH is added followed by a 30-minute incubation at 42 ºC to denature the DNA. 100 µL of bisulfite salt solution is added to the DNA and incubated for 14 hours at 50ºC. Bisulfite treated DNA is purified using Zymogen DNA columns and eluted 20 µl of T1E0.2 8.0 and 1 µl of it is used for

each PCR. PCR was performed with 0.2 μM of each primer and one of the PCR primers was biotinylated to purify the final PCR product using Sepharose beads. The PCR product was bound to Streptavidin Sepharose HP (GE Healthcare Life Sciences), and the Sepharose beads containing the immobilized PCR product were purified, washed and denatured using a 0.2 M NaOH solution and rewashed all using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing, Qiagen). 0.2 μM pyrosequencing primer was annealed to the purified single-stranded PCR product. 10 μl of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Pyrosequencing, Qiagen) following manufacturer instructions (Pyrosequencing, Qiagen). The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software (Pyrosequencing, Qiagen) (Brakensiek, Wingen, Langer, Kreipe, & Lehmann, 2007; England & Pettersson, 2005; Liu et al., 2006).

Analyzed DNA was presented as percent methylation at each of the four TLR4 CpG sites. The four TLR4 methylation variables were all significantly correlated (all *r*s between .48-.68). Thus, the percent methylation at each CpG was averaged to form the TLR4 average methylation score used in all analyses. Using the average of these four highly correlated variables is preferable to examining each methylation site independently to reduce measurement error and decrease the likelihood of Type I error.

Statistical Analysis Plan.

Analyses of Specific Aims. Study hypothesis 1 is a test of path A (Figure 2), and states that heavy drinkers as compared to light drinkers will exhibit greater levels of basal cytokine expression, greater cytokine expression in response to LPS stimulation, and increased TLR4 methylation. First, we examined the main effect of drinking group on cytokine expression using one-way ANOVA. To further explore the relationship between drinking and cytokines and

TLR4, we conducted a series of ordinary least squares (OLS) multiple regressions in which we separately regressed circulating and stimulated cytokines and TLR4 methylation on an alcohol consumption measured by the TLFB, as well as any covariates (i.e., self-report variables that were significantly different between drinking groups). For any significant models, we tested moderators as appropriate, by creating an interaction term in regression models. Moderators were selected based on whether these variables have previously demonstrated a theoretical association with both alcohol use and inflammation, and whether they demonstrated an association with alcohol use in the present sample, given that correlations between alcohol consumption and covariates increase the likelihood of detecting moderation effects (see McClelland & Judd, 1993).

Aim 2 is a test of path B (Fig. 2). We examined whether any of the biological inflammatory variables (e.g., circulating or stimulated cytokines or TLR4 methylation) predicted scores on cognitive measures. We used a series of OLS regressions to examine each cytokine separately as a predictor of the two NIH toolbox measures of interest, including any covariates that demonstrated a significant association with inflammatory variables and theoretical associations with cognitive variables.

For Aim 3, we examined path C (Figure 2) by comparing the heavy and light drinking groups on their performance on tests from our cognitive battery using a one-way ANOVA. As a follow-up, we used a regression to examine whether scores on a continuous measure of drinking predict scores on the cognitive measures. If these relationships were significant, we planned to examine whether the relationship between drinking and cognitive performance was mediated by stimulated or circulating cytokines.

As exploratory hypotheses, we tested whether methylation of CpG sites within TLR4 is associated with circulating and stimulated levels of inflammatory cytokines using simple correlations.

CHAPTER III

RESULTS

Sample Characteristics and Available Data

We collected a total of N=82 subjects (43 recruited as heavy drinkers and 39 recruited as light drinkers). Table 1 presents the means and standard deviations for various demographic variables, drinking measure and other potential covariates. Overall means (and SDs) are included, and these values are also provided for heavy and light drinking groups. All significant group differences are indicated in Table 1. Note that although subjects were screened out for illicit drug use on the phone screen, 10 people reported illicit drug use at least once during the past 90 days on the TLFB. Given that this was not regular use, and to preserve sample size, we allowed these individuals to remain in the analysis. However, one subject reported regular use of an illicit substance, so this individual was also dropped from all analyses.

Table 1

Sample Demographics

Demographic Variable	**Overall Sample (n=82)**	**Heavy Drinkers (n=43)**	**Light Drinkers (n=39)**
	Mean (SD) or Number (%)	**Mean (SD) or Number (%)**	**Mean (SD) or Number (%)**
Age	29.91 (4.5)	30.28 (4.9)	29.53 (4.2)
Gender (male)	39 (47.6%)	27 (62.8%)**	12 (30.7%)**
Race			
American Indian/Alaska Native	1 (1.2%)	1(2.2%)	0 (0%)
Asian	8 (9.5%)	2 (4.4%)	6 (15.4%)
African American	3 (3.6%)	3 (6.7%)	0 (0%)
White	68 (81.9%)	34 (79.0%)	33 (84.6%)
Mixed	3 (3.6%)	3 (6.7%)	0 (0%)
DHQ Age of Drinking Onset	19.42 (2.4)	19.00 (2.3)	19.97 (2.4)
AUDIT Consumption	5.93 (2.9)	7.77 (2.3)***	3.92 (2.1)***
ADS total	6.32 (4.6)	7.8 (4.1)**	4.77(4.6)**

TLFB Drinks/Drinking Day	3.48 (2.5)	4.56 (2.5)***[a]	2.28 (2.0)***[a]
BDI	9.40 (8.1)	10.26 (7.7)	8.47 (8.5)
BAI	6.39 (7.2)	6.08 (6.6)	6.74 (7.9)
TLFB Cannabis Days	22.30 (33.4)	38.93 (37.7)[*a]	12.90 (25.9)[*a]
TLFB Number of Cigarettes	8.18 (33.9)	13.95 (45.9)[a]	1.83 (6.3)[a]
PSQI Sleep Duration	6.83 (1.2)	6.69 (1.3)	6.98 (1.1)
PSS Total	24.5 (8.6)	25.1 (8.8)	23.91 (8.5)
Daily Fruit/Veg	4.53 (3.1)	4.06 (2.6)	5.06 (3.6)
VAEQ Exercise Days/Week	3.54 (2.1)	3.5 (2.0)	3.59 (2.2)

Note. Significant group differences indicated by *** p <.001, **p<.01, * p<.05. [a]Welch's p value used to determine significant group differences due to violation of heterogeneity of variances assumption. DHQ=Drinking History Questionnaire, AUDIT=Alcohol Consumption Identification Test, ADS=Alcohol Dependence Scale, TLFB=Timeline Followback, BDI=Beck Depression Inventory, BAI=Beck Anxiety Inventory, PSQI=Pittsburgh Sleep Quality Index, PSS= Perceived Stress Scale, VAEQ=Voluntary Aerobic Exercise Questionnaire

Regarding availability of data points across subjects, there were 11 subjects for whom we did not collect sufficient plasma to test circulating cytokine levels using the high-sensitivity ELISA kits. In addition, there were 3 subjects for whom we could not draw blood for various reasons (e.g., dehydration), so these individuals only have self-report and cognitive data. Two individuals do not have complete cognitive data due to technical errors occurring during administration of the cognitive batteries.

Cytokine Assay Results

Cell Count. To ensure that differences in cytokines were not due to differences in total number of monocytes, we measured total number of PBMCs, monocytes and lymphocytes in fresh whole blood, as well as post-incubation control and post-incubation LPS stimulated samples. However, we were only able to accurately determine monocyte count in a subset of the sample (n=56), thus normalization of cytokine data for the primary study analyses would have resulted in a significant decrease in sample size. Although the Scepter cell counting method is comparable to Flow Cytometry (Cappione, Crosslet, Thirumalapura & Hoover, 2011), it is not as

sensitive or precise, and is thus less ideal for normalization. Instead of normalizing all data for analyses, we compared Scepter-based cell counts between heavy and light drinking individuals in the subsample for whom cell counts were available (n=56). No significant group differences in PBMCs, monocytes or lymphocytes were observed across any of these sample types. We concluded that any differences in cytokines likely cannot be attributed to differences in total number of monocytes. PBMCs were cryopreserved so that Flow Cytometry could potentially be performed to quantify and differentiate cell populations within the present sample in the future.

High Sensitivity ELISA Data for Circulating Cytokines. Of the 82 subjects included in the study, 11 individuals did not have sufficient plasma and 3 individuals did not provide a blood sample. Thus, there were a total of 68 individuals run on the HS ELISA kits. For each cytokine, two plates were assayed. For IL-6, n=65 (one subject who did not have complete cognitive data was not run at all due to having to re-run numerous samples that failed to be detected on the first run, and 2 outliers [defined as individuals whose data points were more than 3 standard deviations above or below the mean] were removed). For IL-8, n=64 (two subjects without complete cognitive data were not run at all due to having to re-run numerous samples that failed to be detected on the first run, 1 subject [a light drinker] had an IL-8 concentration that was too low to detect, and 1 outlier was removed). For IL-1β, n= 60 (1 subject without cognitive data was not run, and 7 subjects [5 heavy drinkers and 2 light drinkers] had IL-1β concentrations that were too low to detect). For circulating HMGB1, n=67 (1 outlier was removed). Cytokine concentrations are reported in pg/mL

ELISA Data for LPS-Stimulated Cytokines. To extend findings from the circulating cytokine assay, the LPS-stimulated assay was run on a subset of samples (n=64). For IL-8, n=62 (one individual [a heavy drinker] had concentrations that were too low to detect and 1 outlier was

removed). For IL-6, n=60, (3 individuals [2 heavy and 1 light drinker] had concentrations that were too low to detect, and 1 outlier was removed). For IL-1β, n=61 (one individual [a heavy drinker] had concentrations that were too low to detect and 2 outliers were removed). For LPS-stimulated HMGB1, n=57 (2 individuals had concentrations too low to detect, and 5 individuals were not run due to insufficient plasma). Cytokine concentrations are reported in pg/mL.

Correlations Between Outcome Variables and Self-Report Measures

Self-report data were collected on variables that have been theoretically and empirically linked to inflammation, epigenetics, and/or cognitive performance, including age (Chung et al., 2009; Salthouse, 2013), gender (Pai et al., 2006), cardiovascular exercise (Woods et al., 2009), stress (Hart & Kamm, 2002; McEwen & Sapolsky, 1995), sleep (Patel et al., 2009), diet (Holt et al., 2009; Lourida et al., 2013), cigarette (Van der Vaart et al., 2005) and cannabis (Curran et al., 2016; Keen, Pereira, & Latimer, 2014) use, as well as age of onset of regular drinking (Pascual et al., 2014), and depression (McDermott & Ebmeier, 2009; Yirmiya, 2000). We examined correlations between these variables and each outcome variable (circulating cytokines, LPS-stimulated cytokines, TLR4 methylation and cognitive variables). These correlations are listed in Tables 2-4. Note that due to an error in Qualtrics survey programming, 15 of the initial subjects run through the study did not complete measures of BDI, BAI, diet, sleep, exercise or stress.

Table 2

Pearson correlations between circulating cytokines and demographic, psychological and substance use variables

Variable	Circulating IL-8	Circulating IL-6	Circulating IL-1β	Circulating HMGB1

Age	.429**	.326**	.469**	-0.221
Gender (male =1)	0.014[a]	0.078[a]	0.196[a]	-0.005[a]
BDI Total	0.164	0.251	-0.046	0.109
BAI Total	0.154	0.046	-0.014	0.025
DHQ Age Drinking Onset	-0.11	-0.223	0.157	0.046
TLFB Cannabis Days	-0.174	-0.049	- .324*	0.21
TLFB Num Cigs	0.026	-0.154	-0.161	0.078
PSQI Sleep Duration	0.036	-0.111	0.048	0.133
PSS Total	.303*	0.2	-0.048	-0.039
Daily Fruit/Veg Consumption	-.059	-.076	.096	.044
VAEQ Cardio Days/Week	-0.107	-0.151	-0.142	0.147

Note. Significant correlations indicated by **p<.01, * p<.05. [a]denotes point-biserial correlation coefficient. DHQ=Drinking History Questionnaire, AUDIT=Alcohol Consumption Identification Test, ADS=Alcohol Dependence Scale, TLFB=Timeline Followback, BDI=Beck Depression Inventory, BAI=Beck Anxiety Inventory, PSQI=Pittsburgh Sleep Quality Index, PSS= Perceived Stress Scale, VAEQ=Voluntary Aerobic Exercise Questionnaire

Table 3

Pearson correlations between LPS-stimulated cytokines and demographic, psychological and substance use variables

Variable	Stimulated IL-8	Stimulated IL-6	Stimulated IL-1β	Stimulated HMGB1
Age	.030	.091	-.116	-.010
Gender (male =1)	-.004[a]	.037[a]	-.270*[a]	-.153
BDI Total	.325*	.382*	.314*	.100
BAI Total	.026	.080	.038	.304*
DHQ Age Drinking Onset	-.268	-.264	-.263	.096
TLFB Cannabis Days	.082	.059	.127	.110
TLFB Num Cigs	.078	-.061	.132	.021
PSQI Sleep Duration	-.009	-.083	.022	-.284*
PSS Total	.248	.401**	.090	.221
Daily Fruit/Veg	-.096	-.172	-.156	.114
VAEQ Cardio Days/Week	.092	-.209	.029	-030

Note. Significant correlations indicated by **p<.01, * p<.05. [a]denotes point-biserial correlation coefficient. DHQ=Drinking History Questionnaire, AUDIT=Alcohol Consumption Identification Test, ADS=Alcohol Dependence Scale, TLFB=Timeline Followback, BDI=Beck Depression Inventory, BAI=Beck Anxiety Inventory, PSQI=Pittsburgh Sleep Quality Index, PSS= Perceived Stress Scale, VAEQ=Voluntary Aerobic Exercise Questionnaire

Table 4

Pearson correlations between TLR4 methylation, cognitive measures and demographic, psychological and substance use variables

Variable	TLR4 Avg. Methylation	Flanker Inhibitory Control	List Sorting Working Memory
Age	-0.184	-0.056	0.064
Gender (male =1)	-0.158[a]	0.049[a]	0.098[a]
BDI Total	0.1	-0.165	0.126
BAI Total	-0.127	-0.241	-0.086
DHQ Age Drinking Onset	-0.021	-0.037	-0.153
TLFB Cannabis Days	0.041	-0.048	0.01
TLFB Num Cigs	0.045	-0.074	-0.008
PSQI Sleep Duration	0.179	.263*	0.013
PSS Total	0.09	-0.115	-0.057
Daily Fruit/Veg Consumption	.093	-.201	.118
VAEQ Cardio Days/Week	0.193	-0.183	0.167

Note. Significant correlations indicated by **p<.01, * p<.05. [a]denotes point-biserial correlation coefficient. DHQ=Drinking History Questionnaire, AUDIT=Alcohol Consumption Identification Test, ADS=Alcohol Dependence Scale, TLFB=Timeline Followback, BDI=Beck Depression Inventory, BAI=Beck Anxiety Inventory, PSQI=Pittsburgh Sleep Quality Index, PSS= Perceived Stress Scale, VAEQ=Voluntary Aerobic Exercise Questionnaire

Results of One-way ANOVA Tests Comparing Heavy vs. Light Drinkers

After removing all outliers (scores greater than 3 SDs above or below the mean) on measures of inflammation (4 outliers were removed from LPS-stimulated cytokine analyses, and 4 were removed from circulating cytokine analyses), we conducted a preliminary comparison of heavy and light drinking groups on measures of alcohol use, other psychosocial variables, cognitive performance, TLR4 methylation, circulating cytokines and LPS stimulated cytokines. We used one-way ANOVAs, given that some variables are non-normally distributed and ANOVA is robust to violations of the normality assumption (Schmider, Ziegler, Danay, Beyer, & Bühner, 2010). Note that at all variables (i.e., cognitive, self-report, methylation and

cytokines) passed tests of homogeneity of variance (i.e., all Levene's statistics were non-significant), except for TLFB drinks per drinking day, TLFB total cannabis days and TLFB number of cigarettes. For those variables, we conducted the Welch's ANOVA procedure, which is an alternative to the classic ANOVA that can be used when data violate the assumption of homogeneity of variances (Tomarken & Serlin, 1986). Significance is indicated in Table 1 and below by the Welch's p value.

As expected, significant AUDIT consumption score differences emerged for heavy drinkers (M=7.77, SD=2.2) and light drinkers (M=3.92, SD=2.1); $F(1,80)=63.558$, $p<.001$, Cohen's $d=1.785$. In addition, significant ADS total score differences emerged for heavy drinkers (M=7.8, SD=4.1) and light drinkers (M=4.77, SD=4.6); $F(1,78)=9.646$, $p=.003$, Cohen's $d=.695$, and significant TLFB drinks per drinking day differences emerged for heavy drinkers (M=4.56, SD=2.5) and light drinkers (M=2.28, SD=2.0); (Welch's $F(1,77.97)=21.124$, $p<.001$) Cohen's $d=1.029$. Also, significant TLFB number of cannabis days differences emerged for heavy drinkers (M=28.93, SD=37.7) and light drinkers (M=12.90 SD=25.9); (Welch's $F(1,74.65)=5.112$, $p=.027$, Cohen's $d=.506$). Group differences in gender emerged such that there were significantly more males in the heavy drinking group compared to the light drinking group $\chi^2(1, N=82)=8.408$, $p=.004$. No group differences were observed for measures of depression, anxiety, daily fruit and vegetable consumption, days per week of cardiovascular exercise, perceived stress, average nightly sleep duration or age of onset of regular drinking.

Regarding cognitive measures, no significant group differences were observed for age-corrected scores on either of the individual tests. Specifically, Flanker Inhibitory Control $F(1,75)=.189$, $p = .665$, Cohen's $d=.1$, List Sorting Working Memory $F(1,74)=.783$, $p=.379$, Cohen's $d=.206$, Circulating IL-6 $F(1,63)=.009$, $p=.924$, Cohen's $d=.024$, Circulating IL-8

F(1,62)=.466, p =.498, Cohen's *d*=.174, Circulating IL-1β F(1,58)=.034, p =.854, Cohen's *d*=.048, Circulating HMGB1 F(1,65)=.382, p =.539, Cohen's *d*=.154, LPS Stimulated IL-6 F(1,58)=.035, p =.853, Cohen's *d*=.049, LPS Stimulated IL-8 F(1,60)=.361, p =.550, Cohen's *d*=.155, LPS Stimulated IL-1β F(1,59)=.084, p =.773, Cohen's *d*=.075 and LPS Stimulated HMGB1 F(1,55)=1.868, p =.177, Cohen's *d*=.369. In terms of TLR4 methylation, no significant group differences were observed for any of the individual CpGs or the TLR4 average methylation value across the four sites, F(1,73)=.089, p =.767, Cohen's *d*=.07. We also tested for significant group differences in cytokines. No significant differences were observed in either circulating cytokine levels or in LPS-stimulated cytokines. Table 5 shows these between group differences in blood variables (TLR4 methylation, circulating cytokines and stimulated cytokines) and cognitive tests.

Table 5

Summary of Between Group Differences: Blood and Cognitive Variables

	***M* (*SD*)**	
Variable	**Heavy**	**Light**
Flanker Inhibitory Control Score	95.54 (*16.44*)	94.00 (*14.56*)
List Sorting Working Memory Score	103.49 (*12.55*)	100.97 (*12.19*)
Circulating IL-6	.85 (*.48*)	.84 (*.42*)
Circulating IL-8	3.38 (*1.55)*	3.65 (*1.64*)
Circulating IL-1β	.26 (*.31*)	.27 (*.29*)
Circulating HMGB1	63020.10 (*23424.98*)	66602.53 (*21942.57*)
TLR4 Average Methylation	2.21 (*.92*)	2.27 (*.88*)
LPS Stimulated IL-6	545.74 (*604.12*)	575.05 (*611.65*)
LPS Stimulated IL-8	694.83 (*11.24.94*)	545.59 (*816.25*)
LPS Stimulated IL-1β	1562.93 (*1318.15*)	1468.96 (*1210.25*)
LPS Stimulated HMGB1	71538.24 (*37422.35*)	85557.76 (*40013.26*)

Note. SD = standard deviation

Regression Results

To increase power to detect effects, drinking was examined as a continuous measure, using a series of Ordinary Least Squares (OLS) multiple regression models. Given that study hypotheses link alcohol *consumption* (rather than alcohol problems or degree of dependence) to changes in inflammatory signaling, the TLFB drinks per drinking day variable was used rather than ADS or AUDIT, as the TLFB drinks per drinking day variable is the purest consumption measure available in the dataset. Figures 4-7 show the relationships between TLFB drinks per drinking day and each circulating cytokine, and figures 8-11 show the relationships between TLFB drinks per drinking day and each LPS stimulated cytokine.

In the present study, we examined covariates that could at least be theoretically linked to the dependent variable in each analysis, and demonstrated some association with the independent variables in the sample. In the set of models in which cytokines were the dependent variable, covariates were included if they demonstrated significant differences between heavy and light drinking groups, and have a theoretical association with inflammation. Thus, TLFB cannabis days and gender were included as covariates in all models. In all models reported below, slope values are reported as standardized coefficients. Significance was set at $p < .05$. Given the preliminary and exploratory nature of this work, we did not correct for multiple tests. In all models reported below, residuals were approximately normally distributed and homoscedasticity was established, as assessed by a visual inspection of a plot standardized residuals versus standardized predicted values for each model.

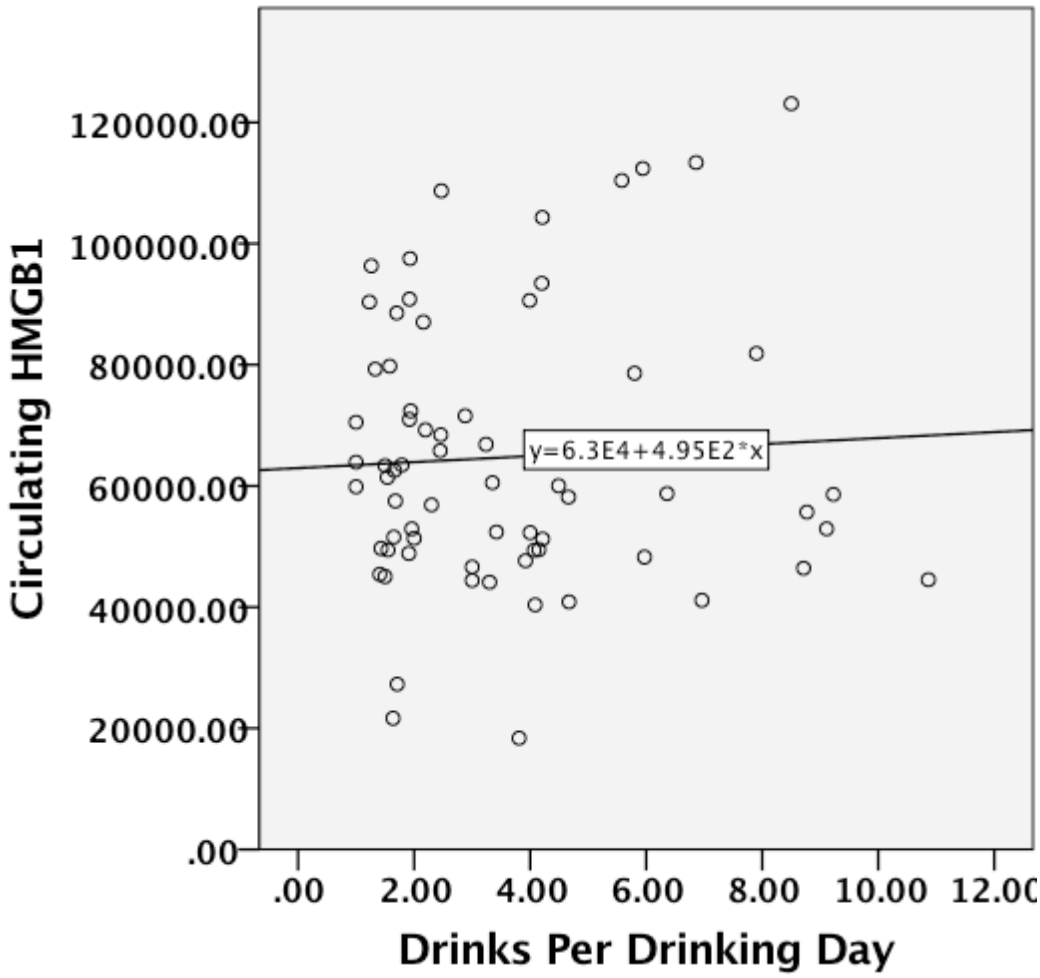

Figure 4. Scatterplot showing the correlation between TLFB drinks per drinking day and circulating HMGB1 (pg/mL).

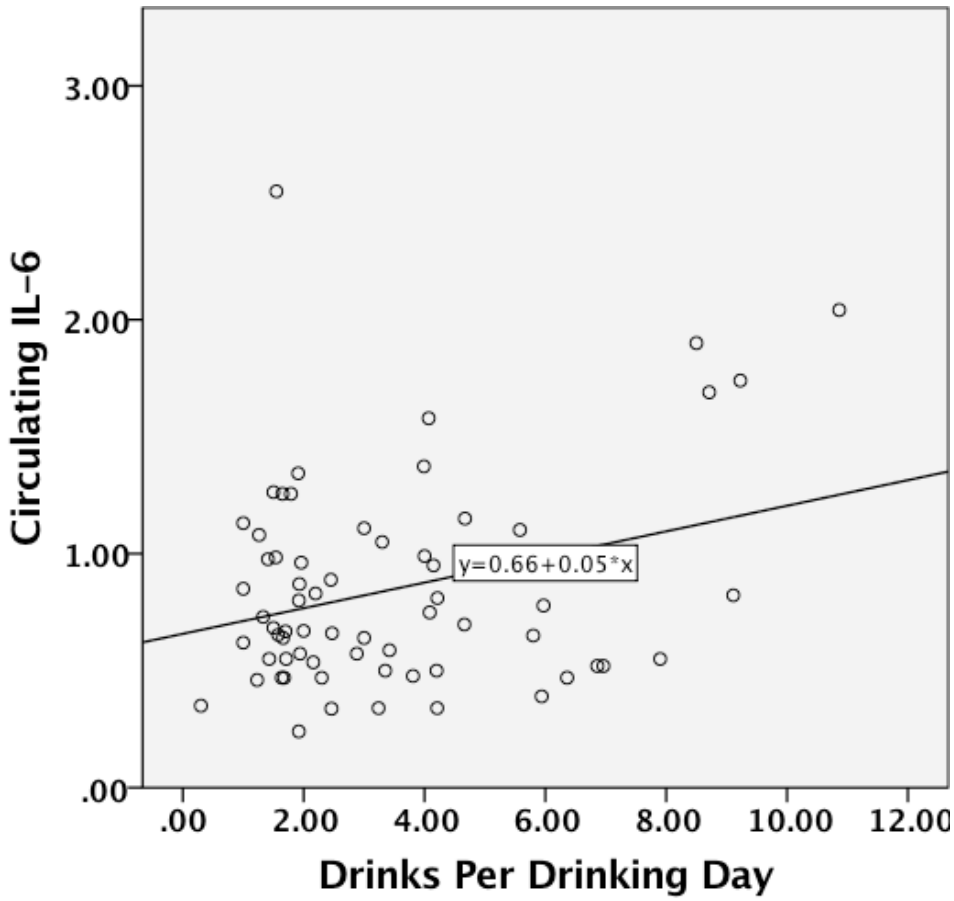

Figure 5. Scatterplot showing the correlation between TLFB drinks per drinking day and circulating IL-6 (pg/mL).

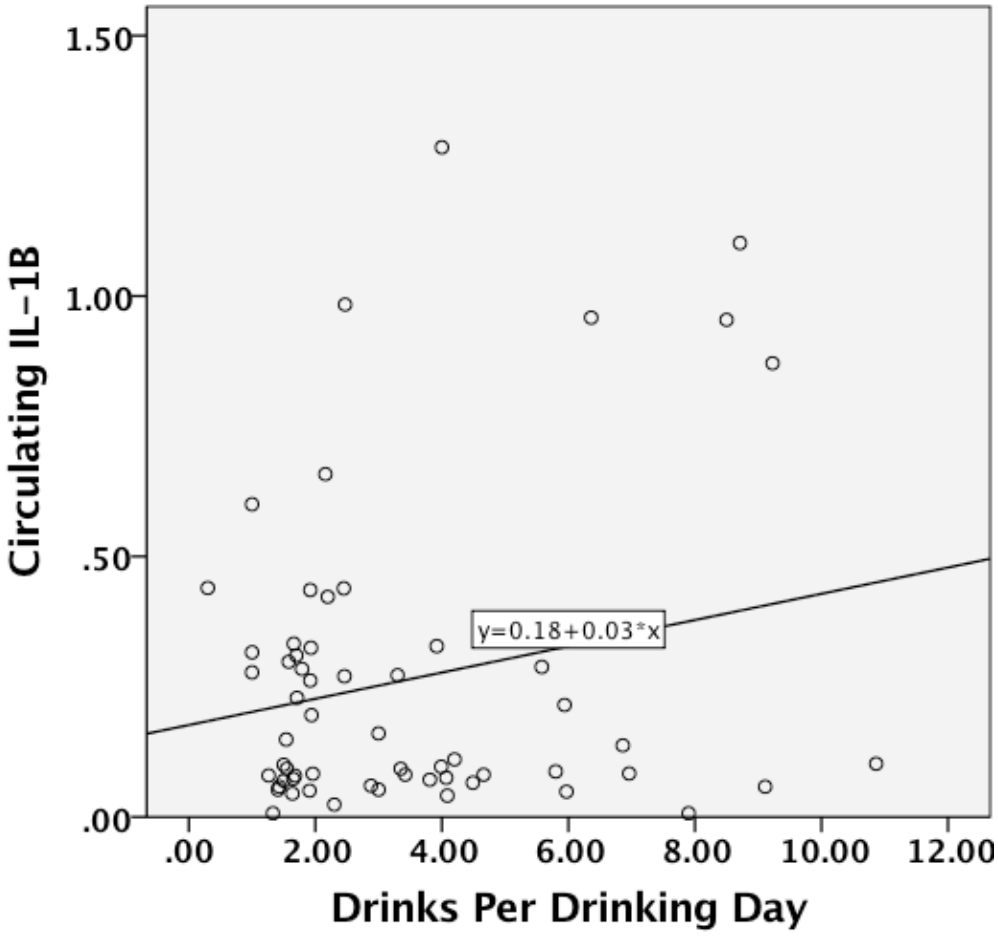

Figure 6. Scatterplot showing the correlation between TLFB drinks per drinking day and circulating IL-1β (pg/mL).

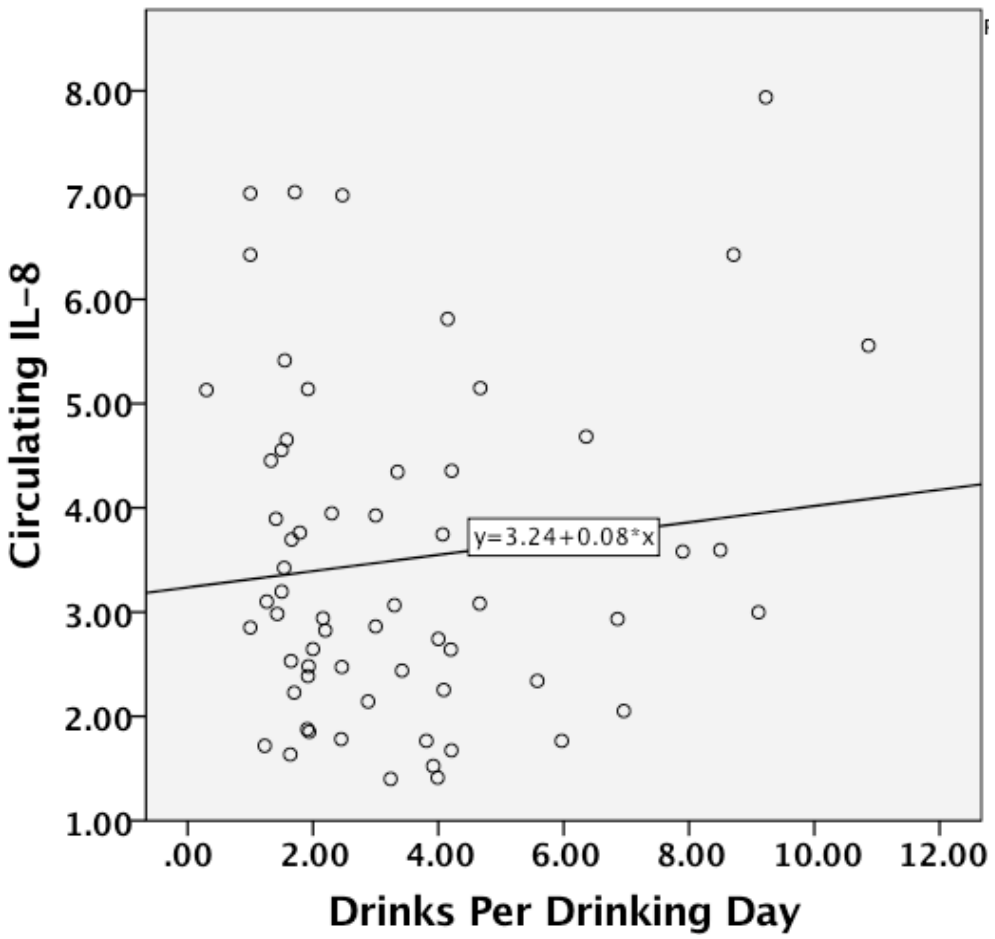

Figure 7. Scatterplot showing the correlation between TLFB drinks per drinking day and circulating IL-8 (pg/mL).

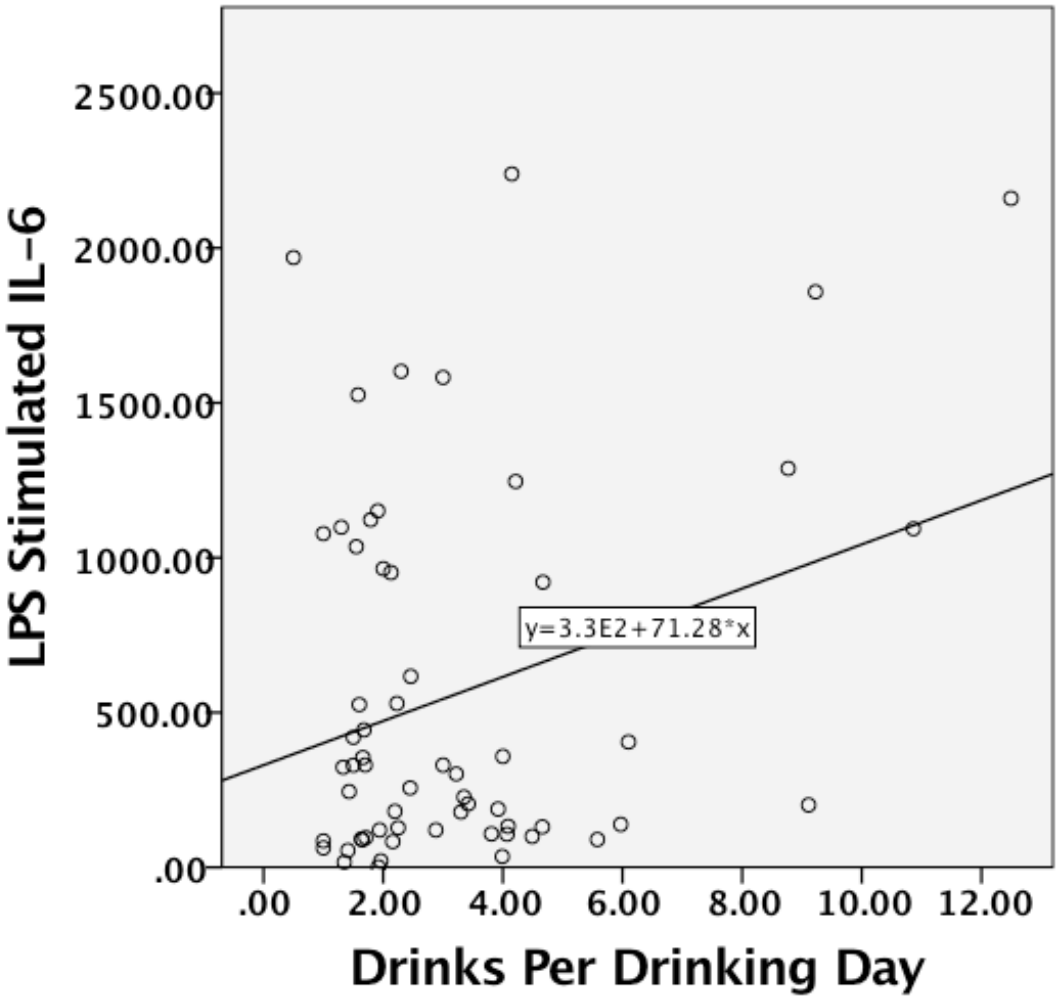

Figure 8. Scatterplot showing the correlation between TLFB drinks per drinking day and LPS stimulated IL-6 (pg/mL).

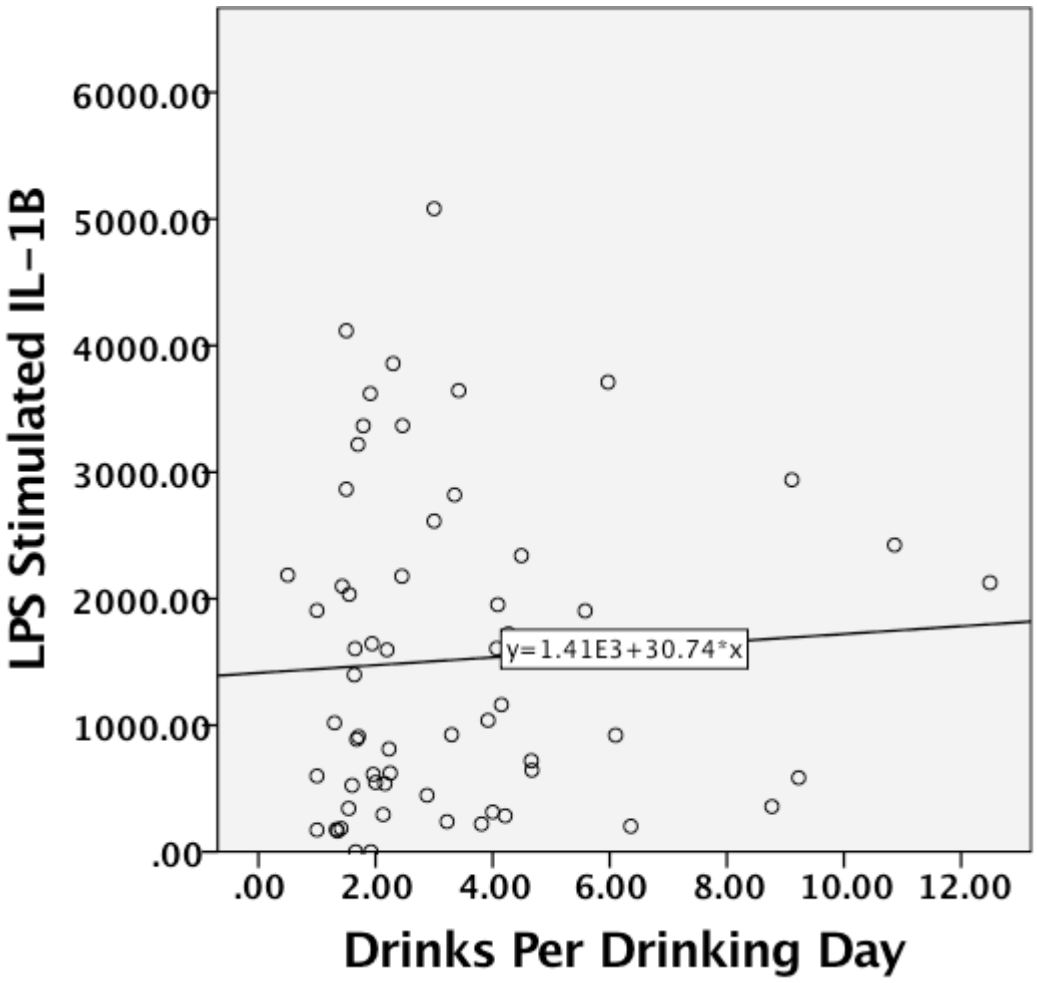

Figure 9. Scatterplot showing the correlation between TLFB drinks per drinking day and LPS stimulated IL-1β (pg/mL).

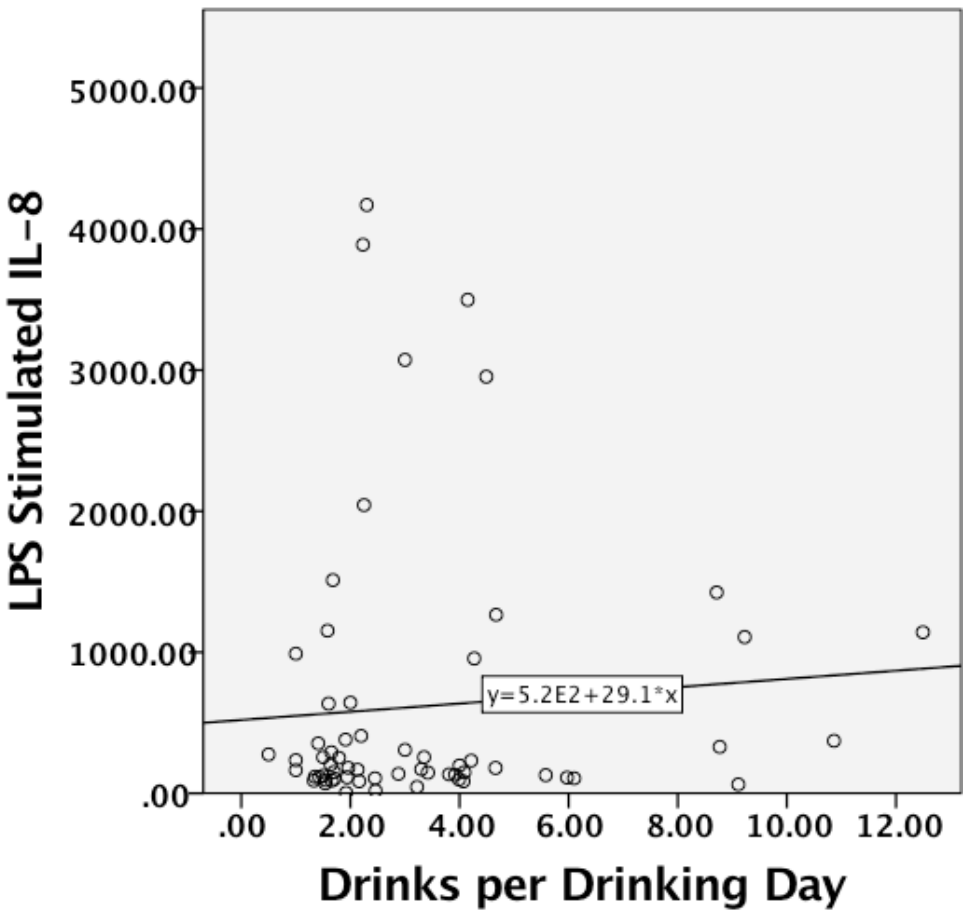

Figure 10. Scatterplot showing the correlation between TLFB drinks per drinking day and LPS stimulated IL-8 (pg/mL).

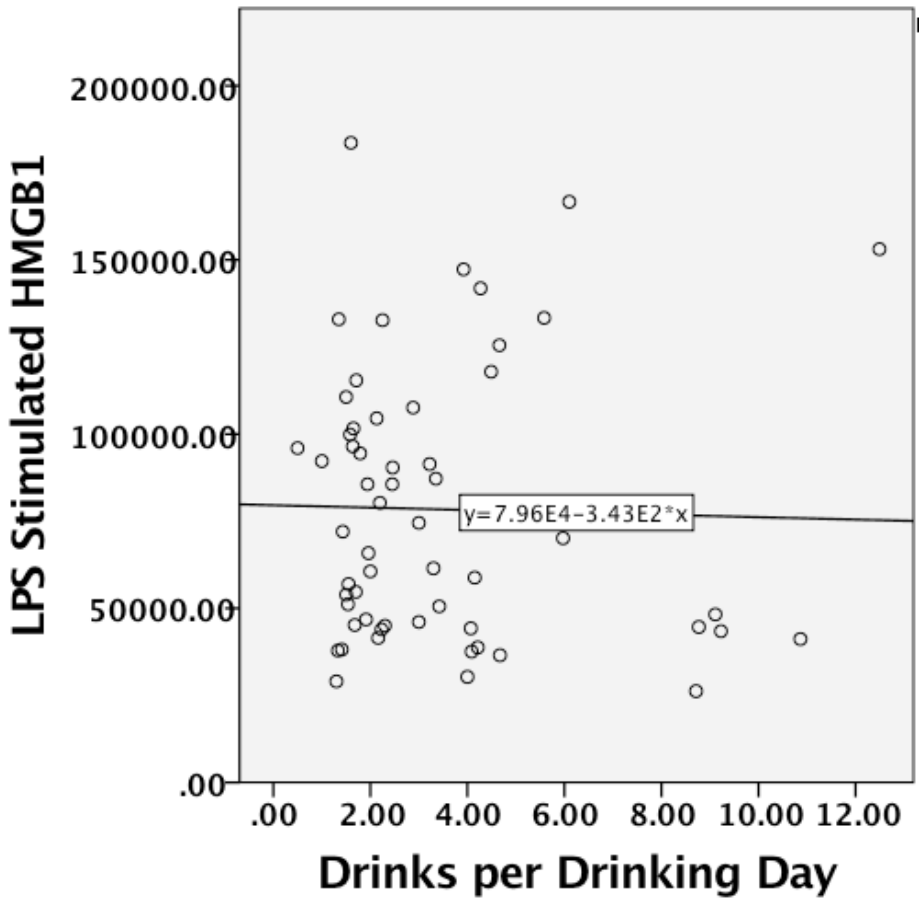

Figure 11. Scatterplot showing the correlation between TLFB drinks per drinking day and LPS stimulated HMGB1 (pg/mL).

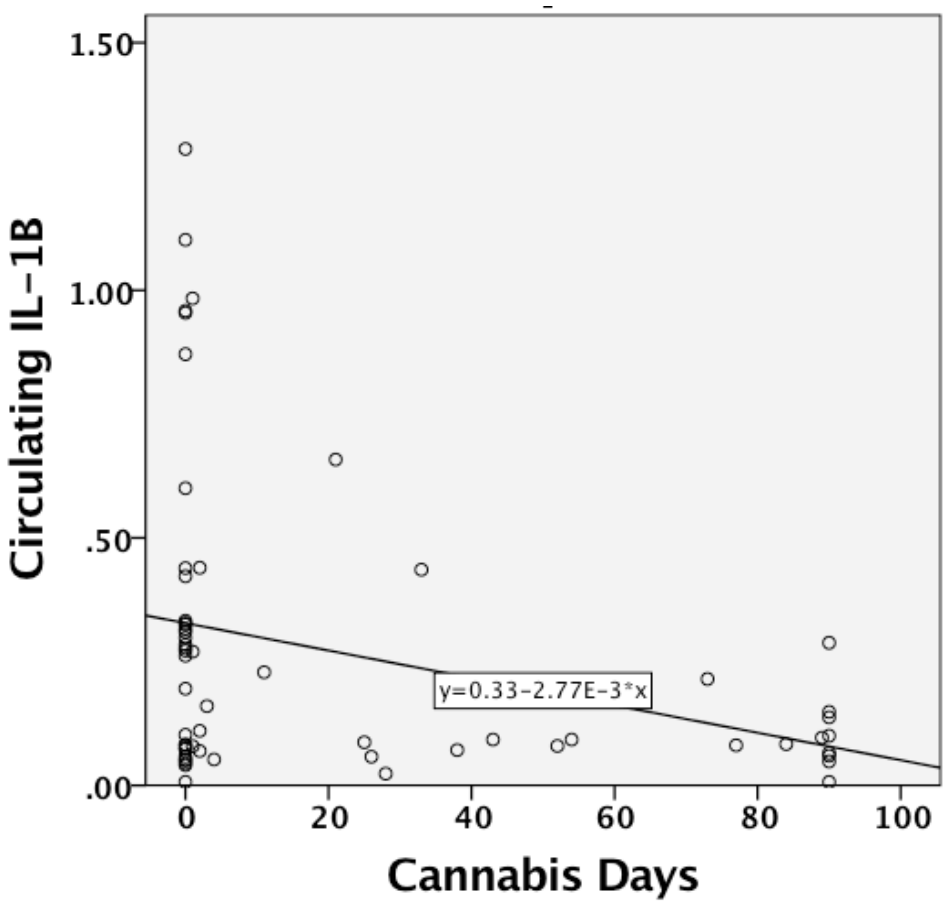

Figure 12. Scatterplot showing the correlation between TLFB cannabis days and circulating IL-1β

First, we aimed to determine whether alcohol use covarying for cannabis use and gender predicted circulating cytokines. We first regressed each cytokine separately on TLFB drinks per drinking day and the specified covariates. In the model in which IL-6 was the criterion, the predictors accounted for 10.5% of the variance in cytokines, F(3, 61)=2.392, p=.077, and inspection of individual regression slopes revealed a significant main effect of TLFB drinks per drinking day, (*b*=.353 *t*(64)=2.529 , *p*=.014).

In the model in which IL-1β was the criterion, the predictors accounted for 23.6% of the variance in cytokines, F(3, 56)=5.782, p=.002. Inspection of individual regression slopes revealed a significant main effect of cannabis days (*b*=-.453 *t*(59)=-3.650, *p*=.001) but no main

effect of TLFB drinks per drinking day. Figure 12 shows the negative association between circulating IL-1β and cannabis use days. In the model in which IL-8 was the criterion, the predictors explained 5.8% of the variance in cytokines, F(3, 60)=1.234, p=.305, and no main effects emerged. In addition, for the model in which circulating HMGB1 was the criterion, the predictors explained 4.9% of the variance, F(3, 63)=1.092, p=.359, and no main effects emerged. Similarly, for the model in which TLR4 average methylation was the criterion, the predictors explained 3.6% of the variance, F(3, 71)=.884, p=.454, and no significant main effects were observed.

Next, we examined whether alcohol consumption predicted LPS-stimulated cytokines, covarying for gender and cannabis days. In the model in which LPS-stimulated IL-6 was the criterion, the predictors explained 9.3% of the variance, F(3, 56)=1.924, p=.136, and inspection of individual regression slopes revealed a significant main effect of TLFB drinks per drinking day (*b*=.338 *t*(59)=2.353, *p*=.022). In the model in which LPS-stimulated IL-8 was the criterion, the assumptions of normality of residuals and homoscedasticity were violated, so the model could not be interpreted. In the model in which LPS-stimulated IL-1β was the criterion, the predictors accounted for 12.1% of the variance, F(3, 57)=2.614, p=.060. Inspection of individual regression slopes revealed a significant main effect of gender (*b*=-.345 *t*(60)=-2.604, *p*=.012). In the model in which LPS-stimulated HMGB1 was the criterion, the predictors accounted for 4.8% of the variance, F(3, 53)=.888, p=.453. No main effects of gender, cannabis days or drinking days emerged.

We also ran two models regressing each of the two cognitive variables of interest (Flanker Inhibitory Control and List Sorting Working Memory) on TLFB drinks per drinking day and cannabis use. Neither of these models were significant.

We also examined whether circulating and stimulated inflammatory cytokines, and TLR4 methylation were associated with cognitive performance on our two measures of interest, by regressing cognitive variables on each inflammatory marker, with covariates selected if they demonstrated a significant correlation with inflammatory variables and were also expected theoretically to be associated with cognitive variables (see Tables 2-4). Covariates included in each model were as follows: for the models in which circulating IL-8 was the predictor, age and PSS total were included; for circulating IL-6, only age was included; for circulating Il-1β, age and TLFB cannabis days were included; for circulating HMGB1 no covariates were included, for stimulated IL-8, BDI total was included; for stimulated IL-6, BDI total and PSS total were included; for stimulated IL-1β, BDI total was included; for stimulated HMGB1, PSQI sleep duration and BAI total were included; and for TLR4 methylation, no covariates were included. None of the inflammatory variables in any of these models significantly predicted cognitive performance.

Finally, as an exploratory aim, we tested whether TLR4 methylation was associated with circulating cytokines. Using simple correlations, we demonstrated that TLR4 average methylation was correlated with circulating HMGB1 (r=.288, p=.021), and that this effect seems to be driven by significant correlations between HMGB1 and two individual CpGs, located at Chr9: 117704398 (r=.289, p=.021), and Chr9: 117704406 (r=.241, p=.055). The partial correlation covarying for age remained significant. No other correlations were observed between TLR4 and circulating cytokines.

Follow-up Analyses

Given that in the above regression models TLFB drinks per drinking day did not significantly predict cognitive performance, and that the proposed mediator (inflammation) was

also found not to predict cognitive performance, we decided not to test whether inflammation mediates this relationship, as originally proposed.

Tests of Moderation. Because TLFB drinks per drinking day and TLFB cannabis days both significantly predicted circulating cytokines, and because cannabis was significantly negatively associated with circulating IL-1β whereas drinks per drinking day was positively associated with IL-6, and given that cannabis days and drinks per drinking days are significantly positively correlated with each other (r=.380, p <.001), we examined cannabis days as a potential moderator of the relationship between drinking and circulating IL-6 and IL-1β. We regressed IL-6 and IL-1β separately on TLFB drinks per drinking day, gender, TLFB cannabis days, and TLFB drinks per drinking day by TLFB cannabis days interaction term. Note that both drinks per drinking day and cannabis days were mean centered for moderation models. In the model in which IL-6 was the criterion, predictors explained 27.3% of the variance, F(4, 60)=5.635, p=.001. Inspection of individual regression slopes indicates a significant cannabis by drinks per drinking day interaction (b=-.434 t(64)=-3.722, p<.001). To further explore the nature of this interaction, we compared cannabis non-users (n=29 subjects with cytokine data) to individuals who had used cannabis at least once over the period covered by the TLFB (n=29 subjects with cytokine data). In the non-users, the partial correlation, covarying for gender was significant (partial r=.724, p<.001), but in the users, the partial correlation covarying for gender was not significant (partial r=-.138, p=.452). Figure 13 illustrates these group differences.

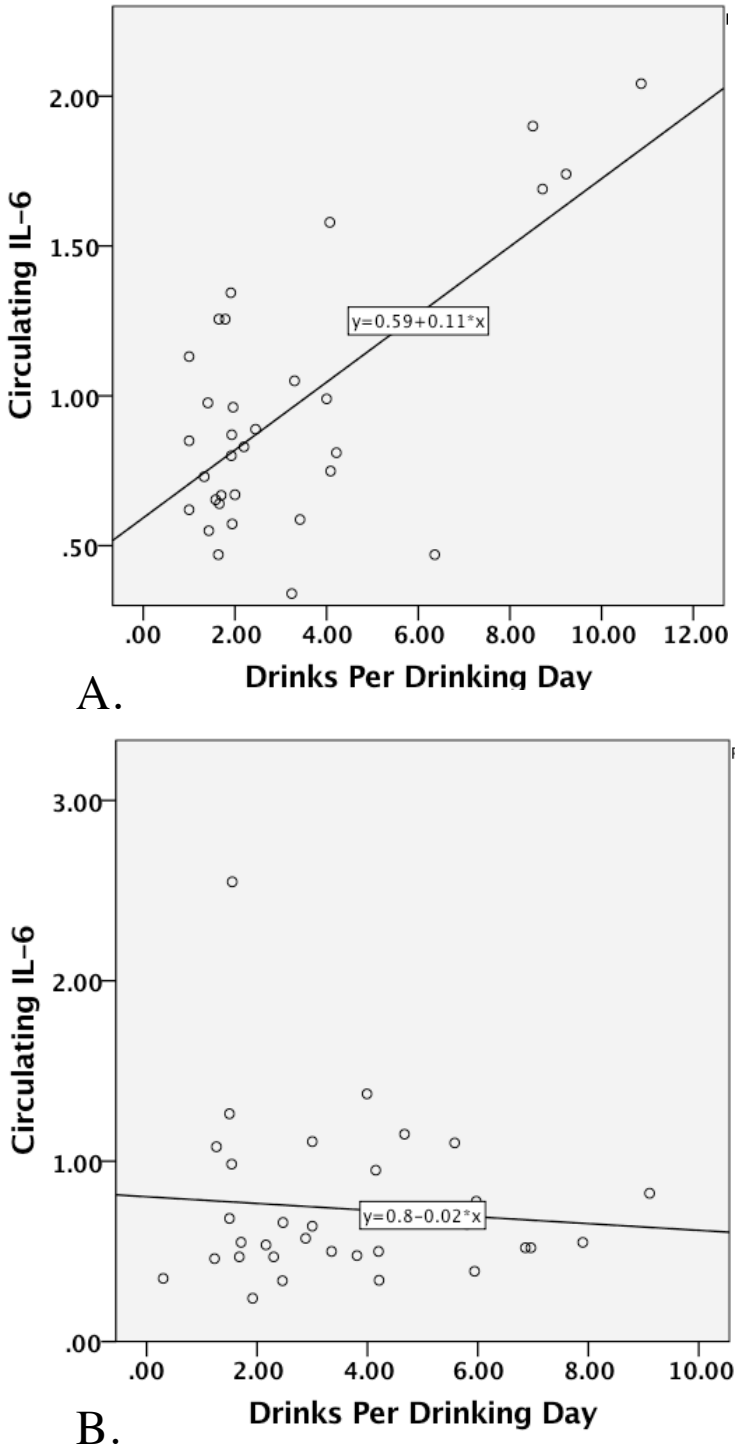

Figure 13. Comparison of the relationship between drinks per drinking day and circulating IL-6 (pg/mL) in subjects who had not used marijuana in the past 90 days (panel A) and individuals who had consumed marijuana in the past 90 days (panel B).

In the model in which IL-1β was the criterion, predictors explained 26.9% of the variance F(4, 55)=5.061, p=.002. Inspection of individual regression slopes indicates no significant cannabis by drinks per drinking day interaction.

Additionally, because gender was associated with LPS-stimulated IL-1β, we also examined whether gender moderates the effect of TLFB drinks per drinking day on stimulated IL-1β (with TLFB cannabis days also included in the model). Gender was contrast coded such that males= 1, females = -1. In this model, the predictors explained 12.1% of the variance F(4, 56)=1.927, p=.119, and only gender (but not the interaction term) was a significant predictor (*b*=-.345 *t*(60)=-2.575, *p*=.013). Finally, given that gender was associated with stimulated IL-1β and cannabis use was associated with circulating IL-1β, we examined whether gender moderates the effect of cannabis use on stimulated IL-1β, including TLFB drinking days in the model. In this model, the predictors accounted for 12.9% of the variance F(4, 56)=2.066, p=.097, and only gender (but not the interaction term) was a significant predictor (*b*=-.340 *t*(60)=-2.552, *p*=.013).

CHAPTER IV

DISCUSSION

This study examined the relationship between alcohol use, circulating and stimulated peripheral inflammatory markers and TLR4 methylation, and cognitive performance in a sample of heavy and light drinkers. Subjects completed all study procedures during a single session involving psychological questionnaire measures, a cognitive battery and a blood draw. Outcomes were compared based on drinking group, as well as using alcohol consumption as a continuous predictor.

Overview of Main Outcomes

Contrary to prior human research suggesting that AUDs are associated with long-term, systemic inflammation indicated by increased circulating cytokines in blood (Achur, Freeman, & Vrana, 2010; Leclercq et al., 2012; Leclercq et al., 2014a), we did not demonstrate any significant differences between the heavy and light drinking groups in terms of circulating or stimulated cytokines. We also failed to observe group differences on cognitive performance or TLR4 methylation. Effect sizes across outcomes may be smaller than anticipated, and it is likely that the two groups were not dissimilar enough on alcohol exposure to detect any differences. It is possible that comparing non-drinkers to alcohol-dependent individuals, treatment seeking individuals with alcohol dependence, or individuals who report a history of chronic alcohol dependence would reveal significant differences in inflammatory mediators or TLR4 methylation. Consistent with that notion, data previously published on this question were often collected from older, more severely dependent patients with greater lifetime alcohol exposure. For example, in our previous work, we demonstrated differences between alcohol dependent

individuals (mean age=48 years) and age-matched controls on TLR4 methylation (Hagerty et al., 2016). Also, a recent study found increased circulating levels of IL-6, IL-8 and IL-1β in alcohol dependent inpatients (mean age=48 years) compared to healthy controls (Leclercq et al., 2014a), suggesting that significant differences may have emerged in the present study if older and/or more severe drinkers had been recruited, perhaps from a medical or inpatient treatment setting.

Regarding cognitive measures, in addition to finding no differences between drinking groups, we failed to demonstrate any associations between alcohol consumption (measured continuously) and cognitive variables, methylation and cognitive variables, or cytokines and cognitive variables. We also examined correlations between cognitive variables and self-report measures (see Table 4). Number of hours of sleep per night emerged as the strongest predictor of performance on the cognitive batteries, which is consistent with the well-established relationship between sleep and cognitive performance (Alhola & Polo-Kantola, 2007). This result underscores the importance of including sleep variables as covariates in any analyses in which cognitive performance is an outcome.

One potential explanation for our lack of findings demonstrating a relationship between alcohol and cognitive performance may be that the measures administered are not sensitive enough to detect subtle differences in cognitive abilities that could be observed in this relatively young, healthy, heavy-drinking population. Given that the neural damage and subsequent cognitive impairments associated with alcohol use are cumulative and increase with increased years of drinking and increased age (Pfefferbaum et al., 1992; Woods et al., 2016), our heavy drinkers, who had an average age of 30.28 years, may have been too young and had not yet experienced sufficient alcohol exposure to demonstrate measurable cognitive deficits. In addition to recruiting older drinkers with a longer alcohol use history for future studies, this problem

could also be addressed by administering cognitive batteries that measure more complex cognitive processes and may be more sensitive to alcohol-related impairment, such as the Iowa Gambling Task, which measures risky-decision making (Kim, Sohn, & Jeong, 2011), the Trail-Making test, which involves multiple brain systems and measures attention and set switching (Day, Celio, Lisman, Johansen, & Spear, 2013), or the Stop Signal Alcohol Cue Task, which examines the ability to inhibit a pre-potent response in the context of craving-inducing alcohol cues (Karoly, Weiland, Sabbineni, & Hutchison, 2014).

Although we failed to demonstrate significant group differences in circulating or stimulated cytokines, when alcohol consumption was considered continuously, drinks per drinking day was positively associated with circulating IL-6. This is consistent with prior work demonstrating that circulating IL-6 is correlated with AUD severity (Leclercq et al., 2014a). We explored cannabis use as a moderator, given that recent studies have suggested that cannabinoids may have profound effects on immune system function and inflammation, both peripherally and centrally (for review, see Klein, 2005). In the moderation model, significant main effects of drinking and a significant interaction between cannabis use and drinks per drinking day emerged. The direction of this interaction is such that lower cannabis use is associated with a stronger relationship between alcohol consumption and circulating IL-6. Specifically, among individuals who did not consume cannabis during the 90-days immediately prior to the study, there was a strong positive relationship between alcohol consumption and IL-6, but in those who did consume cannabis, there was no relationship. These results are consistent with findings from human studies demonstrating that cannabis use is associated with reductions in IL-6 (Keen et al., 2014).

Of note, drinks per drinking day was also found to be a significant predictor of LPS-stimulated IL-6. It has been suggested that heavy drinking may increase sensitivity of cells to LPS-stimulation among individuals with alcoholic liver disease (Schäfer et al., 1995) and following an acute binge alcohol administration (Bala et al., 2014). Our findings are consistent with this prior work. In addition, we observed that stimulated IL-6 was correlated with stress and depression (see Table 2). These results are consistent with prior research demonstrating the role of stress and mood symptoms in influencing LPS-stimulated cytokine release. For example, in rats, a prior stressor was shown to sensitize LPS-induced production of IL-6 and IL-1β (Johnson, O'Connor, Daek, Stark, Watkins & Maier, 2002). In humans, chronic stress has also demonstrated an association with greater LPS-stimulated cytokine production (Davis, Zautra, Younger, Motivala, Attrep & Irwin, 2008). Depression has also been associated with immune dysfunction including altered responses to LPS. For example, severity of mood disturbance has been positively correlated with LPS-induced cytokine production in humans (Yirmiya, 2000), and LPS has been shown to increase depression-like behavior in mice (Frenois et al.,2007).

Notably, cannabis use was not a significant predictor of any of the LPS-stimulated cytokines, which suggests that cannabis may serve to decrease basal levels of inflammation in humans, but does not appear to play a major role in impacting acute responses to an immune challenge. In neonatal rat cortical microglial cells, however, tetrahydrocannabinol (THC; one of the primary compounds in recreational and medical cannabis) decreased LPS-induced mRNAs for various cytokines including IL-6 and IL-1β, with IL-6 demonstrating particular sensitivity to THC (Puffenbarger, Boothe, & Cabral, 2000). THC was also found to decrease LPS-induced IL-6 release from mouse macrophages in cell culture (Chang, Lee, & Lin, 2001).

For circulating IL-1β, we found significant effects of cannabis use but not alcohol use. Interestingly, in the moderation model, the significant main effect of cannabis use remained, but the alcohol by cannabis interaction was not significant. This suggests that the impact of cannabis use on IL-1β may be more important than the impact of alcohol consumption. Similar to IL-6, we found that cannabis use was negatively associated with circulating levels of IL-1β, which is also consistent with the literature (Klein, 2005). However, we found no relationship between alcohol or cannabis use and LPS-stimulated IL-1β, which is counter to findings from a rodent study showing that cannabinoids prevent LPS-induced production of IL-1β in brain tissue (Chung, Bok, Chung, Baik, & Jin, 2012) and that THC decreases LPS-induced mRNA for IL-1β (Puffenbarger et al., 2000). Also, a cannabinoid receptor agonist was found to attenuate LPS-induced increases in IL-1β in rat brain and plasma (Roche, Diamond, Kelly, & Finn, 2006). Our unexpected findings suggest that although cannabis has been shown to impact LPS-stimulated cytokines in animals, the relationship between cannabis and LPS-stimulated inflammatory markers may be more complicated in human peripheral tissue.

Notably, we did observe a main effect of both gender and depression predicting LPS-stimulated IL-1β, which is consistent with existing evidence. We found that gender did not moderate the relationship between alcohol and stimulated IL-1β or cannabis and stimulated IL-1β, indicating that gender does not influence the relationship between substance use and stimulated IL-1β, but should be included as a covariate in subsequent studies of stimulated IL-1β production. Previously, gender differences have been observed in LPS-induced production of cytokines including IL-1β, such that males showed a greater response than females (Aulock et al., 2006). However, in the present study, females demonstrated greater LPS-induced IL-1β than men. Given that estrogen has been found to modulate LPS-induced cytokine secretion (Asai et

al., 2001), and that we did not control for day of last menstrual cycle among our female participants, our unexpected findings may be due to differences in estrogen levels. Alternatively, this pattern of results may be related to our relatively low power or the fact that significant differences in gender composition were present between heavy and light drinking groups. In summary, given the link between estrogen and inflammatory processes, future studies should not only include gender as a covariate, but should also control for the day of last menstrual cycle in female subjects.

Notably, we also observed a correlation between LPS stimulated IL-1β and BDI score (Table 3). This finding is consistent with increasing research supporting the critical role of inflammation and inflammatory cytokines in the pathophysiology of depression (Miller, Maletic, & Raison, 2009; Raison, Capuron, & Miller, 2006). Administration of a pro-inflammatory stimulus can result in depression-like behavioral syndromes (e.g. increases in depressed mood, anxiety, fatigue, mental confusion, and psychomotor slowing (Brydon, Harrison, Walker, Steptoe, & Critchley, 2008), and depression severity has been positively correlated with LPS-induced cytokine production in humans (Yirmiya, 2000). The present results combined with the existing literature on the relationship between depression and inflammation support the inclusion of mood variables as covariates in studies examining inflammatory responses to an immune challenge.

Counter to our hypotheses, circulating IL-8 was not associated with alcohol consumption or cannabis use in the present study. Increased plasma IL-8 has been previously shown in alcohol dependent patients (Hill, Marsano, & McClain, 1993), however the individuals in this study were suffering from alcoholic hepatitis, or liver inflammation, which occurs in some patients as a result of chronic heavy drinking (Lucey, Mathurin, & Morgan, 2009). Another study

demonstrated increased IL-8 among alcohol dependent inpatients compared to healthy controls (Leclercq et al., 2014a). The subjects in the present study did not have significant chronicity of alcohol use, and none reported alcohol-related liver problems or prior inpatient hospitalization, which may explain our lack of findings for IL-8. Further, although there is some evidence for an indirect link between cannabinoid signaling and IL-8 (Mormina, Thakur, Molleman, Whelan, & Baydoun, 2006), the nature of this potential relationship is not well understood. These results suggest that compared to other cytokines, IL-8 may not play a primary role in inflammatory signaling in substance use disorders.

Unexpectedly, we also failed to observe any significant relationships between circulating or stimulated HMGB1 and alcohol use. These results indicate that although HMGB1 is implicated in alcohol-related neuroimmune signaling in humans and animal models (Crews et al., 2013; Zou & Crews, 2014), and serum HMGB1 is induced in alcoholic liver injury (Ge et al., 2014), neither circulating nor stimulated HMGB1 is significantly altered in relatively young, healthy, heavy drinkers. Indeed, even among individuals with alcoholic liver injury, HMGB1 is elevated, but the cytokine response to HMGB1 appears unaltered, suggesting that HMGB1 is not a primary contributor to peripheral inflammation, even in the context of alcoholic liver disease (Laursen et al., 2016). In the brain, however, HMGB1 has been implicated in alcohol withdrawal (Whitman, Knap, Werner, Crews & Breese, 2013) and was shown to mediate alcohol-related neuroimmune signaling (Crews et al., 2013; Zou & Crews, 2014). Thus, although prior evidence suggests that HMGB1 is involved in the pathophysiology of alcohol dependence, our data indicate that it may play a more important role in the brain rather than the periphery.

Additionally, alcohol consumption measured continuously did not significantly predict TLR4 methylation in the present sample. These results were unexpected, given our prior findings

that TLR4 was hypermethylated in alcohol dependent subjects compared to controls (Hagerty et al., 2016). However, our previous study examined brain tissue samples. Thus, it is possible that the relationship between alcohol use and peripheral TLR4 methylation is less straightforward. In addition, the sample in our previous work was older and more severely alcohol dependent. It is possible that peripheral TLR4 would show significant differences between non-drinkers and older, chronically alcohol dependent individuals. Future research should thus examine blood levels of TLR4 methylation among an older, more severe drinking population.

Finally, as an exploratory aim, we examined whether TLR4 methylation was associated with circulating or stimulated cytokines. The only significant relationships that emerged were between two TLR4 CpGs (located at Chr9: 117704398 and Chr9: 117704406) and circulating HMGB1. Given that TLR4 promoter methylation is associated with TLR4 silencing in several cell types (Takahashi, Sugi, Hosono, & Kaminogawa, 2009; Zampetaki et al., 2006), TLR4 methylation is likely suggestive of decreased TLR4 expression, which could imply decreased activity of the TLR4-mediated inflammatory signaling cascade. HMGB1 contributes to the regulation of innate immune gene expression through activating TLR4 (Crews et al., 2013). Thus, one interpretation of our exploratory finding is that HMGB1 may increase to compensate for decreased TLR4 expression resulting from TLR4 methylation. However, given that these results are extremely preliminary, and further, that the majority of studies examining alcohol-induced HMGB1/TLR4 inflammatory signaling have been conducted in rodent brain tissue (e.g., Crews, Qin, Sheedy, Vetreno, & Zou, 2013; Zou & Crews, 2014), the possible relationship between TLR4 methylation and HMGB1, particularly in human peripheral tissue, remains unclear.

Overall, the most notable result from the present study is the moderation by cannabis consumption of the relationship between alcohol consumption and circulating IL-6. This finding warrants a more in-depth discussion of the mechanism(s) by which cannabinoids (which are the compounds isolated from cannabis, of which the most well-studied are tetrahydrocannabinol [THC], cannabidiol [CBD] and cannabinol [CBN]) may exert anti-inflammatory properties in the brain and periphery, particularly in the context of heavy alcohol use. In recent years, CBD has shown promise as an anti-inflammatory agent. Briefly, CBD has demonstrated anti-inflammatory effects peripherally (Burstein & Zurier, 2009), as well as in the brain. CBD is a lipophilic molecule that can cross the blood-brain barrier (Devinsky et al., 2014), and microglia have been shown to express cannabinoid receptors (Cabral & Marciano-Cabral, 2005), suggesting that cannabinoids, including CBD, can directly impact neuroinflammation. Notably, CBD and THC have been shown to regulate microglial activity and cytokine production (including IL-6 and IL-1β) in response to inflammatory signals, likely via actions on the TLR4-mediated NFkB inflammatory signaling pathway (Levy, Kozela, Rimmerman, & Pietr). In one study, a single dose of CBD was shown to protect against neuroinflammation following an LPS stimulation in mice (Ruiz-Valdepeñas et al., 2011). CBD has also been shown to reduce neuroinflammation in the context of several neurodegenerative disorders and other inflammatory conditions. CBD reduced microglial activation in a rat model of Alzheimer's disease (Martín-Moreno et al., 2011), and specifically suppresses IL-1β (Esposito et al., 2007). CBD also attenuates inflammation in a viral model of multiple sclerosis (Mecha et al., 2013) and reduces the immune response in a rat model of pneumococcal meningitis (Barichello et al., 2012). With respect to alcohol use disorders, our data suggest that CBD may similarly serve to decrease inflammation in the brain

and periphery. To date, few empirical studies have examined the influence of cannabis and alcohol together on inflammatory signaling.

Limitations and Future Directions

The primary statistical limitation in the present study was power. Given the loss of subjects due to problems that arose during the blood protocols and Qualtrics data collection, we were underpowered for both group comparisons and regression analyses. For this reason, significant findings should be interpreted with caution until replicated with adequately powered samples. This caution is particularly important regarding interpretation of the cannabis moderation effect, given the large sample size needed to detect interaction effects (McClelland & Judd, 1993). In addition, our lack of significant group differences should also be interpreted with caution until a replication can be conducted. Thus, an important next step will be to collect data on 15-20 additional participants to increase power to detect moderate effects in both group comparisons and regression analyses.

Regarding the circulating cytokine findings, there are several limitations to note. Importantly, although alcohol likely does produce peripheral inflammation, given that alcohol dependence is associated with elevated plasma levels of LPS and cytokines in humans (e.g., Achur et al., 2010; Leclercq et al., 2012; Leclercq et al., 2014a), it is possible that alcohol's effect on inflammatory markers in the periphery does not play a primary role in inducing its neuroinflammatory effects. Briefly, peripheral LPS can lead to neuroinflammation in the brain via inducing IL-1β (Ren & Torres, 2009), which can then cross the blood brain barrier through entering at circumventricular organs (e.g., area postrema, vascular organ of the lamina terminalis or subfornical organ), active transport, inducing PG production in endothelial cells of the cerebral vasculature that then enters the brain parenchyma, and by activating vagal fibers

(Engblom et al., 2002; Maier, Goehler, Fleshner, & Watkins, 1998; Quan & Banks, 2007). However, peripheral inflammation has been found to induce neuroinflammation when there is a very large increase in plasma Il-1β, such as in the case of an acute endotoxin administration (Qin et al., 2008), but we did not observe such elevated circulating 1L-1β levels in the subjects in the present study. Thus, it is unlikely that there was significant inflammatory signaling between the periphery and brain in these subjects.

Perhaps more importantly, alcohol itself crosses the blood-brain barrier and directly activates TLR4 (Alfonso-Loeches, Pascual-Lucas, Blanco, Sanchez-Vera, & Guerri, 2010; Lewis et al., 2013), thereby promoting inflammatory signaling in the brain. In addition, alcohol promotes a neuronal GABA/glutamate imbalance, which is associated with excitotoxicity and cell-death, which induces local inflammation in the brain (Mon, Durazzo, & Meyerhoff, 2012). The presence of ethanol in the brain also induces oxidative stress and produces acetylaldehyde, which may lead to cellular damage and consequent neuroinflammation (Almansa et al., 2009; Sun & Sun, 2001). Taken together, the evidence suggests that these direct effects of alcohol on the brain likely dominate the peripheral inflammatory effects that occur as the result of alcohol use, and that peripheral inflammation may not be a major source of neuroinflammation in AUDs. Relatedly, our lack of correlation between peripheral inflammatory markers and cognitive performance may be due, in part, to the fact that there is not a strong correlation between peripheral and neural inflammation in the alcohol users in the present sample, owing to either their relatively low level of chronicity and symptom severity, or because neural and peripheral inflammation are truly not significantly associated in alcohol use disorders.

However, some recent work has suggested that peripheral inflammation may play a role in the development and maintenance of alcohol use disorders, specifically through alcohol-

induced gut-permeability and intenstinal dysbiosis, which refers to the development pathogenic bacteria in the gut, and has been shown to not only exacerbate depression, anxiety and craving, but possibly contribute to the symptoms of alcohol addiction through interacting with the stress system, disrupting normal sleep processes and interfering with social interaction (de Timary, Stärkel, Delzenne, & Leclercq, 2017). Not surprisingly, given that the gut is a major source of immune factors, considerable human research has already demonstrated the critical role of gut microbiota in influencing psychiatric disorders and promoting aberrant behavior (Dinan, Stilling, Stanton, & Cryan, 2015; Foster & Neufeld, 2013). Further, alcohol dependent subjects with dysbiosis have demonstrated greater severity of AUD symptoms compared to those without dysbiosis (Leclercq et al., 2014b). Thus, an important next step is to further examine the possible role of gut permeability and intestinal dysbiosis in alcohol-induced inflammation through incorporating gut microbiome sample collection into studies of alcohol use and inflammation.

Another limitation of the present study is related to the fact that although we demonstrated no differences in monocytes between the heavy and light drinking groups, we did not normalize cytokine data for analysis to account for number of monocytes. Normalization of LPS-stimulated cytokine measurement to individual monocyte counts has been found to significantly alter results (Aulock et al., 2006). Thus, monocytes should be considered when measuring cytokine levels. It is also worth noting that in the present study, we used the Millipore Scepter 2.0 for cell counting, which differentiates cells based on their size (diameter). Although cell counting using this method is comparable with cell counts obtained from Flow Cytometry, the Scepter method has less sensitivity and precision in terms of population cell count (Cappione, Crosslet, Thirumalapura & Hoover, 2011). Thus, it would be ideal to use Flow Cytometry to generate cell counts and then normalize cytokine data based on these counts. Given that we have

cryopreserved PBMCs from all subjects, a potential next step may be to perform Flow Cytometry analyses on these data for normalization in subsequent analyses.

Regarding TLR4 methylation, although methylation sites were selected based on proximity to the transcription start site and location within a TLR4 regulatory region, we measured methylation in a small region of the gene, and it is possible that methylation within another region would confer stronger effect sizes. In addition, although we assume that greater methylation decreases TLR4 expression, and that this decrease in expression blunts inflammatory signaling, further studies are needed to directly measure the relationship between TLR4 methylation and expression in human brain and peripheral tissue. Future work is also needed to establish concordance between TLR4 methylation in buccal and brain tissue. However, it is promising that emerging research has demonstrated an association between buccal and brain tissue methylation (Smith et al., 2015), and we previously found that methylation of CpGs (including CpGs in TLR4) differed significantly between AUD and control subjects and was consistent across brain and buccal cells (Hagerty et. al, 2016). Finally, bisulfite sequencing methods including the pyrosequencing technique we employed in the present study cannot differentiate between methylcytosine variants (e.g., 5-hydroxylmethylcytosine) (Huang et al., 2010), which may have distinct regulatory effects (e.g., Wen et al., 2014; Wu et al., 2011). This fact further contributes to the uncertainty of the relationship between TLR4 methylation and expression, and should be addressed in future work.

Summary and Conclusions

Contrary to initial hypotheses, the present study did not demonstrate statistically significant differences between heavy and light drinkers on cognitive measures, inflammatory markers or TLR4 methylation. Given the prior literature supporting these relationships, these

results may be attributable to the lack of AUD severity/chronicity and relatively young age of the heavy drinking sample. Alternatively, the fact that our data indicated no relationships between drinking and peripheral IL-8, IL-1β, HMGB1 or TLR4 methylation could be interpreted as preliminary support for the lack of convergence between peripheral inflammatory signaling and neuroinflammation in the context of alcohol use disorders. Regardless of interpretation, the present results indicate the need for further research to better understand the role of inflammatory signaling in the brain and periphery in alcohol use disorders.

Notably, when alcohol consumption was examined as a continuous predictor, we did observe significant associations between alcohol consumption and both circulating and stimulated IL-6, as well as moderation by cannabis use of the relationship between alcohol and circulating IL-6. These findings provide initial support for our original hypotheses, and reinforce the need for future research in this area. Ultimately, convergence between human and animal studies of alcohol consumption and inflammatory signaling across various tissue types would provide the most compelling evidence for better understanding the relationship between inflammation, neurocognition and alcohol use, including explicating the possible role of peripheral immune signaling.

Taking into account the limitations of the present study, particularly power, our significant results should be interpreted with caution. However, our lack of group differences should also be interpreted cautiously, given the potentially small effect sizes across these outcomes. To summarize, in light of previously demonstrated associations between alcohol, cannabis and inflammation, both the expected and unexpected findings from the present study should be tested for replication in a larger sample comprised of older, more severely alcohol-dependent individuals. The present results can serve to inform the selection of inflammatory

markers to test in future analyses (e.g., peripheral IL-6 appears to be associated with alcohol consumption, while IL-8 may be less critical). Overall, despite the preliminary nature of these results and the need for replication, our findings provide promising initial data to inform future investigations, with the goal of ultimately leveraging knowledge of the role of inflammatory signaling in alcohol use disorders to develop more effective treatments focused on novel targets within the immune system.

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