Brain immune signaling in response to binge-like drinking

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THESIS

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List of abbreviations

ALDH	Acetaldehyde dehydrogenase
ALT	Alanine transaminase
ANOVA	Analysis of variance
AUD	Alcohol use disorder
BAL	Blood alcohol levels
BEC	Blood ethanol levels
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
CBP	CAMP response element-binding protein - binding protein
CCL2	Chemokine (C-C motif) ligand 2
CCL3	Chemokine (C-C motif) ligand 3
CCL4	Chemokine (C-C motif) ligand 4
CCL5	Chemokine (C-C motif) ligand 5
CDC	Center for Disease Control
CIE	Chronic intermittent ethanol
CREB	CAMP response element-binding
CSF-2	Colony stimulating factor 2
CSF-3	Colony stimulating factor 3
CV	Coefficient of variation
CXCL1	Chemokine (C-X-C motif) ligand 1
CXCL8	Chemokine (C-X-C motif) ligand 8
DBS	Deep brain stimulation
DC	Detergent compatible
DID	Drinking in the Dark
DSM-5	Diagnostic and Statistical Manual of Mental Disorders 5th edition
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration
FSS	Forced swim test
G-CSF	Granulocyte - colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDID-1	High Drinking in the Dark line 1
HDID-2	High Drinking in the Dark line 2
HMGB1	high mobility group box 1
I.G.	Intragastric
I.P	Intraperitoneal
IFN-γ	Interferon gamma
IHDID-1	Inbred High Drinking in the Dark line 1
IL-10	Interleukin 10
IL-12p40	Interleukin 12p40
IL-12p70	Interleukin 12p70
IL-13	Interleukin 13
IL-15	Interleukin 15
IL-17A	Interleukin 17A
IL-18	Interleukin 18
IL-1α	Interleukin 1 alpha

IL-1β	Interleukin 1 beta
IL-2	Interleukin 2
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-9	Interleukin 9
KG	Kilograms
LPS	Lipopolysaccharide
MAP	Managed alcohol programs
MCP-1	Monocyte chemoattractant protein 1
MG	Milligram
MIP-1α	Macrophage inflammatory protein 1 alpha
MIP-1β	Macrophage inflammatory protein 1 beta
ML	Milliliter
MRNA `	Messenger ribonucleic acid
NAc	Nucleus accumbens
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NMDA	N-methyl-D-aspartate
N-PrOH	N-propanol
NSDUH	National Survey on Drug Use and Health
OFC	Orbitofrontal cortex
OH	Ohio
PA	Pennsylvania
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
pg	Picograms
POC	Proof of concept
PST	Pacific Standard Time
RANTES	Regulated upon Activation, Normally T-expressed, and Presumably Secreted
SEM	Standard error of the mean
SFWM	Superior frontal white matter
SN	Substantia Nigra
STAT3	Signal transducer and activator of transcription 3
TLR	Toll-like receptor
TLR4-KO	Toll-like receptor 4 knock out
TMS	**Transcranial magnetic stimulation**
TNF-α	Tumor necrosis factor alpha
vmPFC	ventromedial prefrontal cortex
VTA	Ventral tegmental area
WHO	World Health Organization

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Abstract

Binge drinking is associated with the genetic risk of developing alcohol use disorder (AUD) and is considered a strong predictor of AUD diagnosis. In 2023 the National Survey on Drug Use and Health reported that 61.4 million individuals 12 years or older reported binge drinking alcohol in the past month. Excessive alcohol drinking produces lasting disruptions in inflammatory signaling in the brain and body. Chronic alcohol consumption leads to increased pro-inflammatory proteins which, over time, led to tissue damage. Chronic alcohol is associated with an increased risk of cancer and inflammation often is associated with cancer development and progression. These studies suggest immune signaling and inflammation may represent a target for reducing excessive drinking and its harmful consequences. Apremilast, a PDE4 (phosphodiesterase type 4) inhibitor with known anti-inflammatory actions has been shown to reduce binge-like ethanol intake in mouse models of genetic risk for drinking to intoxication (inbred high drinking in the dark mice, iHDID). The purpose of this thesis was to test the effects of apremilast administration on early-stage (four days) and initial (one day) binge-like drinking and nucleus accumbens (NAc) cytokine levels in iHDID-1 mice. We also measured whether apremilast affected blood ethanol levels and accumbens cytokines levels. First, 72 male and female iHDID-1 mice underwent a 4-day DID (drinking in the dark; 2hr days 1-4) with 20% ethanol or water. Mice received apremilast (i.p. 40 mg/kg) or vehicle control prior to day 4. Following DID, peri-orbital blood was collected for determination of blood ethanol concentration (BEC). Brains were collected and processed for multiplex cytokine immunoassay. We found apremilast treatment reduced ethanol ($p<0.0001$) and water intake ($p<0.01$) compared to vehicle controls. Results of a 3-way ANOVA (fluid, treatment, sex) demonstrated a significant main effect of fluid for 12/23 cytokines (IL-1α, IL-1β, IL-3, IL-6, IL-9, IL-17A, IFN-g, TNF-

α, eotaxin, MIP-1α, RANTES and KC; all p-values < 0.05), where ethanol treated mice exhibited higher expression levels. To test the effects of an initial exposure to ethanol (with or without apremilast treatment), we carried out a 1-day DID using iHDID-1 mice and collected BECs and brain tissue from the accumbens as described in experiment 1. We found that apremilast decreased ethanol and water intake after initial ethanol exposure, with no observed effects on BEC. The multiplex immunoassay results revealed no significant effect of treatment or fluid type. We were not powered to test sex-specific effects of initial ethanol exposure on cytokine expression. This work shows that NAc cytokine levels increase starting as early as 4 days after binge-like ethanol drinking. Apremilast did not ameliorate the alcohol-induced changes on cytokine expression in either experiment. A one-time initial 2-hour binge exposure to ethanol is not sufficient to alter cytokine expression in the accumbens. Here, this thesis provides additional evidence supporting apremilast as a method of reducing ethanol intake, and the role ethanol can have on early-stage cytokine expression.

Thesis overview

This thesis is broken down into 4 chapters. Chapter 1 discusses the introduction and relevant background information. Chapter 2 discusses the methodology used for the two experiments described. Chapter 3 discusses the results of the 4 day and 1 day DID experiments. Chapter 4 finishes up the discussion by describing the limitations and conclusions appropriate for the work presented here.

Chapter 1. Introduction

1.1 Alcohol use and its impact

The World Health Organization (WHO) describes alcohol (ethanol) as a toxic drug with psychoactive properties. Alcohol's effects are widely distributed through the body and when consumed leads to effects on nearly every organ in our body. The most recent National Survey on Drug Use and Health (NSDUH) from 2023 reported that 224 million individuals 12 years or older had consumed alcohol in their lifetime (Meich et al., 2024). Insight into past year usage reported by the NSDUH showed that within the same population, 177 million people had reported alcohol use (Center for Behavioral Health Statistics and Quality, 2025).

The most recent national report on the annual costs of excessive alcohol consumption (from 2006-2010) estimated that excessive drinking costs $249 billion (Sacks et al., 2015). The economic costs were calculated using 26 different criteria that could be attributed to excessive alcohol consumption such as hospitalization, nursing homes, prevention and research, impaired productivity at work, property damage, amongst others (Sacks et al., 2015). Sacks and colleagues defined excessive alcohol as binge drinking or heavy drinking. Binge drinking is defined by the NIAAA as four or more drinks for women and five or more for men per session. Heavy drinking was defined as

eight or more drinks for women and 15 or more drinks for men per week. Interestingly, Sacks et al., (2015) found that binge drinking accounted for three fourths of the total economic cost of annual excessive alcohol consumption ($191.1 billion of $249 billion). Diving further, the authors also determined that the median cost of binge drinking per state was 2.6 billion (Sacks et al., 2015). From an economic perspective, it is crucial to find ways to decrease alcohol use disorder (AUD) and alcohol misuse. To find answers, we can study different risk factors that lead to someone developing an AUD.

Binge alcohol drinking increases the risk of developing AUD (Tavolacci et al., 2019). Briefly, AUD is defined by the *Diagnostic and Statistical Manual of Mental Disorders 5th edition* (DSM-V) as the presence of at least two symptoms within a 12-month period:

- Had times when you ended up drinking more, or longer than intended?
- More than once wanted to cut down or stop drinking, or tried to, but could not?
- Spent a lot of time drinking? Or being sick or getting over other aftereffects?
- Wanted a drink so badly you could not think of anything else?
- Found that drinking – or being sick from drinking – often interfered with taking care of your home or family? Or caused job troubles? Or school problems?
- Continued to drink even though it was causing trouble with your family or friends?
- Given up or cut back on activities that were important or interesting to you, or gave you pleasure, to drink?
- More than once gotten into situations while or after drinking that increased your chances of getting hurt (such as driving, swimming, using machinery, walking in a dangerous area, or having unsafe sex)?

- Continued to drink even though it was making you feel depressed or anxious or adding to another health problem? Or after having had a memory blackout?
- Had to drink much more than you once did to get the effect you want? Or found that your usual number of drinks had much less effect than before?
- Found that when the effects of alcohol were wearing off, you had withdrawal symptoms, such as trouble sleeping, shakiness, restlessness, nausea, sweating, a racing heart, or a seizure? Or sensed things that were not there?

For the DSM-V, the severity of AUD is determined by how many of the above symptoms are met, with mild = 2-3 symptoms, moderate = 4-5 symptoms, and severe = >6 symptoms.

In the 2023 NSDUH survey, it was found that 61 million of 134.7 million alcohol users 12 years or older reported binge drinking in the past month. This report also showed that 10.2% of the US population (≥ 12 years old) met the criteria for AUD, and annually there were around 178,000 deaths involving alcohol. Alcohol consumption has been shown to lead to various health problems on the brain (central) and body (periphery). In 2007, a group of researchers were determined to assess the carcinogenicity of beverages that contain alcohol (Baan et al., 2007). This group of researchers concluded that alcohol is carcinogenic in humans (Baan et al., 2007). According to data from CDC WONDER 2025, a database used for the analysis of public health data, nearly 45% of all deaths between 2015-2019 were attributed to liver disease involved alcohol in individuals ≥ 12 years of age. Together, these reports demonstrate that misuse of alcohol is an unmet public health problem.

1.2 Approved medication for AUD

AUD is a lifelong problem (Nieto et al., 2021). Although there are pharmacological treatments for AUD, few people are offered treatment, and these

medications don't work for everyone. Acamprosate, naltrexone, and disulfiram are the three medications for AUD that have been approved by the Food and Drug Administration (FDA). The first FDA approved medication for AUD was disulfiram in the 1950s. Disulfiram acts through inhibition of the acetaldehyde dehydrogenase (ALDH), an enzyme that converts acetaldehyde into acetate during alcohol metabolism (Swift and Aston., 2015, Yahn et al., 2013). This inhibition leads to side effects such as diarrhea, dizziness, vomiting, nausea or tachycardia (Swift and Aston., 2015, Yahn et al., 2013). These negative side effects are meant to deter patients from consuming alcohol. This medication appears to work best in patients who can comply with medication scheduling (Swift and Aston., 2015).

Naltrexone is a non-specific opioid receptor antagonist that was approved by the FDA in 1994 for patients experiencing alcohol dependence as a method of relapse prevention (Yahn et al., 2013). Injectable naltrexone (190 mg or 380 mg) was shown to decrease the amount of heavy drinking days by 25% in a randomized controlled trial (Anton 2008). This clinical trial emphasized the importance of an individual's ability to obtain abstinence before receiving treatment for at least four days (Anton 2008). Naltrexone treatment comes with several side effects such as nausea, headaches, fatigue or vomiting, which makes it less desirable for patient compliance or adherence (Anton 2008). Some of these side effects are limited to the initial stages of treatment, or if taken after eating (Anton 2008). If disulfiram and naltrexone lead to noncompliance of medication adherence, another option offered is acamprosate.

Acamprosate (calcium acetyl homotaurine) was approved for treatment in the US as of 2004 (Yahn et al., 2013). Acamprosate has a mixed efficacy on alcohol craving, relapse, and abstinence as reviewed by Yahn et al., (2013), but has been shown to be safe, cost-effective, and has few side effects. There is mixed evidence on the therapeutic mechanism of acamprosate, but one prominent theory is its role of

modulating N-methyl-D-aspartate (NMDA) receptors (Yahn et al., 2013). Alcohol dependence can lead to increased neuronal excitability over time, leading to negative withdrawal symptoms. Some of these negative symptoms include anxiety, insomnia, increased risk of seizures or epileptic episodes (Tsai & Coyle., 1998, Yahn et al., 2013). Alcohol is known to decrease NMDA receptor activity, acamprosate modulates similar receptor activity, and has been shown to reduce negative withdrawal symptoms suggesting that acamprosate could work for some individuals (Tsai & Coyle., 1998, Yahn et al., 2013).

As described above, there are a few different treatment options for AUD, but they are underutilized. According to a review by Rehm et al., (2016), primary care physicians are not screening for the beginning stages of AUD or alcohol misuse, until it has become severe. The authors suggest this could be due to a physician's lack of education on early signs or symptoms or lack of financial reimbursement (Rehm et al., 2016). Koob (2024) describes a similar underutilization of treatments, suggesting that lack of knowledge, screening, referral to treatment, treatment facilities availability and stigma as explanations.

Interestingly, a qualitative study in a broad spectrum of individuals who were non-treatment seeking and alcohol dependent, found that there were various barriers to receiving treatment (Wallhed Finn, et al., 2014).

As a caveat, this study occurred in Stockholm, but Wallhed Finn et al., (2014) provide insights that are relevant. The authors found that the stigma of being associated with the stereotype "alcoholic" meant that they would be perceived as a failure or deprived of their social networks if they sought out treatment. Wallhed Finn et al., (2014) also reported participants 18-34 years of age misunderstand current treatments as a pathway to lifetime abstinence. Typically, people in the study had negative associations with pharmacological treatment and would have preferred only psychotherapy.

Participants were generally aware of disulfiram and felt that it led to a lack of autonomy. Acamprosate and naltrexone were not as well known, but did have less stigma association compared to Disulfiram.

1.2.1 Harm reduction

So far, the status of alcohol and its effects on the general population as well as the treatment options have been discussed. The goal of these existing treatment options is to reduce harm. Harm reduction is not a novel idea. In fact, as harm reduction methodology was being implemented more generally, a highly cited *Addictive Behaviors* publication from 1996 described what harm reduction means (Marlatt 1996). Marlatt describes harm reduction as a compassionate set of principles and procedures that were designed by people with a history of drug use themselves, based on public health principles. Marlatt (1996) explains harm reduction in contrast to the two competing theories of addiction at the time, the moral model and the disease model. These two theories were often contradictory, but both idealized the idea of lifetime abstinence, which as mentioned above, has led to a decrease in treatment seeking. Principles of harm reduction shift the focus from the user to the consequences of addictive behaviors.

The field has embraced and refined the concept of harm reduction over the last 2 decades. Chan et al., (2022) described harm reduction as a method of reducing the negative consequences on an individual's health, social life, and the economic costs of substance misuse and addiction. Chan et al., (2022), describes managed alcohol programs (MAP) that provide safe alcohol consumption environments in combination with social services such as primary care services, food, or housing. MAP treatment promotes supervised alcohol intake as patients aim to decrease their drinking. Crucially, the authors point out abstinence as not being required to achieve harm reduction, and any decrease in heavy drinking can lead to improved health and wellness outcomes (Chan et al., 2022).

A large part of harm reduction for AUD treatment includes evidence based behavioral treatment options. Behavioral treatments help people with AUD develop skills to reduce their drinking, help them manage their emotions and stress, build foundational support systems, and develop coping strategies when cue-induced triggers occur (NIAAA). These treatments include (from NIAAA alcohol treatment resource):

- Cognitive-behavioral therapy: Therapy focused on identifying cues that could lead to drinking and changing the thought processes surrounding their behavior.

- motivational enhancement therapy: Therapy that helps a patient strengthen their motivation to reduce or stop drinking alcohol, such as identifying pros and cons.

- contingency management approaches: Therapy that uses positive reinforcement to help patients achieve their treatment goals.

- 12 step facilitation therapy: Groups (like Alcoholics' Anonymous) that help the patient in a structured process with support from others.

- mindfulness-based relapse prevention uses cognitive behavioral therapy and mindfulness practices to help respond to physical and emotional cues

These treatments are described briefly here with little nuance but are incredibly important in combination with other interventions.

1.2.2 Deep Brain Stimulation

Deep brain stimulation (DBS) has been historically used to treat Parkinson's disease or epilepsy in targeted brain areas, and more recently was incidentally found to also help reduce alcohol craving (Bach 2023, Müller et al., 2016, and Voges et al., 2013). DBS is not FDA approved for AUD but was initially reported to effectively reduce alcohol consumption in a patient seeking DBS treatment in the nucleus accumbens (NAc) for an anxiety disorder (Kuhn et al., 2007).

DBS of the NAc in male patients suffering from treatment-resistant AUD has successfully reduced drug craving and promoted abstinence (Heinze et al., 2009, Müller

et al., 2016, Voges et al., 2013). Although the initial reports were promising, the surgery is highly invasive and study recruitment requirements were not met, resulting in a discontinuation of their funded project. Thus, finding less or non-invasive options for treatment resistant patients is critical for harm reduction. One option that is less invasive, deep repetitive transcranial magnetic stimulation (Deep TMS), was recently shown to reduce craving in patients with AUD using a double-blind, randomized and sham-controlled clinical trial (Selim et al., 2024). Although the exact mechanism that leads to reduced craving is not fully understood, the authors reported a long-lasting reduction in craving up to 3 months with only 5 follow up sessions after the initial 15 treatment sessions. Deep TMS research is ongoing and a recent review by Harmelech et al., (2021), discusses the benefits for obsessive-compulsive disorder and smoking cessation. Importantly, deep TMS with a H7 coil (the furthest depth according to Harmelech et al., 2021) is only able to target the PFC and the anterior cingulate cortex, which is a tradeoff for the technology, non-invasive but limited.

One take away from brain stimulation studies is the wide range of ways we can target AUD to find treatments, from different brain areas to different types of stimulation or medication. To test and discover further treatment options, we can look to animal models.

1.3 Animal models for AUD are important and necessary for development of AUD treatments

Research using animal models has been critical for improving our understanding of pharmacological therapies for addiction. In the 1980s, T. Cicero posited that a good animal model for AUD would require 6 different criteria:

1. Animals should be consuming alcohol orally.
2. Pharmacologically relevant levels of blood ethanol concentrations should be reached.

3. The animals should be consuming ethanol for its effects regardless of how it tastes, smells or if it provides caloric benefits.
4. Ethanol and its association should be related to positive reinforcement.
5. Extended or chronic ethanol administration should lead to observable tolerance or metabolic changes.
6. chronic ethanol should also lead to physical dependence (Cicero T. 1980)

In practice, all these criteria are not always necessary, but they do provide a set of principles to better understand the value of an animal model for AUD. Because animal models cannot completely recapitulate the human condition of AUD, researchers have developed several assays to model different aspects or stages of AUD, such as binge/intoxication, preoccupation/craving and withdrawal/negative effect (Koob and Volkow, 2010). A good animal model will also have construct, face, and predictive validity.

Briefly, criteria for the validity of animal models were described for modelling human psychopathology in the 1980s (Willner, 1986). In this seminal paper, predictive validity of a model is defined as when the performance on a test can predict the performance of the behavior being modelled (Willner, 1986). Face validity was described as the similarity of treatment and symptomology in the assay being used (Willner, 1986). Willner (1986) described construct validity as requiring two criteria:

1. The constructs being studied are similar between animals and people.
2. The assessments being measured and modelled must be a core part of the disorder.

1.3.1 Early stages of AUD: binge-like drinking

Following the criteria described by Cicero, Rhodes et al., (2005) set out to develop a model of voluntary ethanol self-administration that would result in

pharmacologically relevant blood ethanol levels. In this study, Rhodes et al. (2005) developed a behavioral assay for C57BL/6J (B6) mice that did not require sweetening ethanol or restricting food and water, to get the mice to drink intoxicating levels. To achieve this, Rhodes tested whether B6 mice would drink 20% ethanol in higher quantities if they had limited access (2-4 hours) and it was offered 3 hours into the mouse's active cycle (during the dark phase of the light/dark cycle). This assay occurred over four days and led to most mice voluntarily achieving intoxicating levels of ethanol (blood ethanol concentrations (BECs) > 0.8 mg/mL). This assay is called "Drinking in the Dark" (DID). The advantage of having a four-day behavior that led to intoxication without much training meant there was now a high through-put assay to better understand the genetics, circuits, transcriptomics, and neurobiological effects of drinking to intoxication, and pharmacologically relevant drugs could be tested quickly and efficiently (Rhodes et al., 2007). A follow up study by Rhodes et al., (2007) used DID to test ethanol consumption of 12 different strains with accessible behavioral, physiological, and morphological data from the Mouse Phenome Database from Jax is available. Ethanol intake (g/kg) was found to be highest in C57BL/6J mice and lowest in the DBA/2J mice (Rhodes et al., 2007). B6 mice drank 6.2 – 6.9 g/kg of 20% ethanol during the four-hour DID session (Rhodes et al., 2007). B6 mice were the only mice to reach intoxicating BEC levels (measured in mg/mL), with the next closest strain only reaching 50 milligrams in 100 mL (mg%) BEC, 30 mg% below intoxication.

Using DID, Crabbe et al., (2009) created a line of mice that were selectively bred for reaching high BECs (> 80 mg%). The High Drinking in the Dark line 1 (HDID-1) mice were selectively bred to create an animal risk model for intoxication, and by the Selection generation 9 , these mice were consistently reaching above 1.0 mg/mL BECs (Crabbe et al., 2009). This did not end here, another line of mice was also developed alongside HDID-1 mice, with the same phenotypic outcome. HDID-2 mice were able to

reach > 1.4 mg/mL by Selection generation 20 in a similar manner that HDID-1s did at the same selection (Crabbe et al., 2014). Use of selective breeding created two separate strains of mice from a genetically diverse background, which provides evidence of genetic correlation of excessive alcohol consumption (Crabbe et al., 2009, 2012a, 2012b, 2014, Barkley-Levenson and Crabbe, 2014).

So far, we have discussed DID and HDID mice as animal models of binge drinking and intoxication, but how does this fit in with the T. Cicero criteria? DID and HDID animal models meet Cicero criteria (1) they drink alcohol orally, and (2) they reach relevant BEC levels, (3) HDID mice have been shown to drink ethanol despite quinine (a bitter tastant) adulteration (Crabbe et al., 2011), (4) ethanol's association with positive reinforcement are not tested directly using the DID, (5) tolerance and metabolic changes have been tested using the DID (see Crabbe et al, 2009, 2012a, 2012b), (6) chronic ethanol and physical dependence was not tested directly in any of the studies mentioned.

Do these animal models meet the validity criteria? The DID does exhibit predictive validity, whereby this model is able to provide predictions about different pharmacological outcomes. These models are also like symptoms exhibited by those who binge drink, such as motor incoordination (Rhodes et al., 2007). These assays are modeling core parts of AUD (binge drinking and genetic risk signatures), thus exhibiting construct validity. Although, it must be noted that this early model of binge drinking does not address or lead to similar outcomes that one would expect of chronic alcohol use (Crabbe et al., 2009).

1.3.2 Bender – Model for excessive alcohol consumption

Animal models were also developed to induce physical dependence (bender and chronic intermittent ethanol, CIE) and to model the consequences of chronic excessive alcohol consumption on the brain and body.

One aspect of chronic alcohol consumption in humans is the development of negative consequences in the liver. To study this risk factor of AUD, intragastric oral gavage provided an efficient way to create liver problems in B6 mice (Bertola et al., 2013). This behavioral assay includes ten days of liquid diet containing 5% ethanol plus a single excessive binge-like oral gavage (5 g/kg body weight) session. The effect of this assay leads to dangerously high BECs (>500 mg%), as well as increased levels of alanine transaminase (ALT), which is a biological measure of liver damage from alcohol (Bertola et al., 2013). Another assay using i.g. as a route of administration, administered ethanol daily for 10 days (Qin and Crews, 2012). Although the two timelines of i.g. administration of ethanol mentioned above are different, they both result in high levels of intoxication, modelling the effects of excessive alcohol consumption in humans. This model lacks translation for self-administration of alcohol but does allow researchers to test other questions related to the chronic effects of alcohol on the liver and other tissues in the body.

This model of alcohol consumption doesn't address the T. Cicero criteria very well, except for criteria (2), which states that pharmacologically relevant levels of BECs are reached. Despite this, this model remains a good model for its validity. This model provides face validity for liver damage caused by excessive alcohol consumption. Chronic alcohol use does have construct validity, it provides similar outcomes between people and animals, as well as modeling a core part of the consequences of alcohol misuse, liver damage and disease. The predictive validity of this model is not discussed here.

1.3.3 Dependence – vapor CIE

Another aspect of AUD modelled in animals is dependence and withdrawal symptoms.

The escalation of drinking and dependence. Lopez and Becker (2005) developed chronic intermittent ethanol vapor exposure (CIE) in combination with a limited access 2 bottle choice assay to induce dependence, withdrawal symptoms and escalation. To achieve this, mice were subjected limited access to ethanol (2hr/day) until a baseline was established. Then, the mice received either continuous (64hrs) or intermittent (16hrs/day) ethanol vapor exposure. Finally, mice underwent 5 days of ethanol self-administration to test if their behavior changed (Lopez and Becker, 2005). This assay showed the importance of intermittence (withdrawal from ethanol for 8hrs) on increases in ethanol self-administration. This assay was further expanded on by testing intensity and duration of ethanol exposure (Griffin et al., 2009).

Chronic vapor CIE meets the following Cicero (1980) criteria, (1) animals consume ethanol orally, (2) this model leads to pharmacologically relevant BECs, (6) vapor plus self-administration leads to withdrawal symptoms suggesting physical dependence. Mice exhibit similar symptoms as people who are dependent or experience withdrawal effects from alcohol offering face validity. Finally, this model only meets half of the construct validity criteria defined by Willner (1986), modeling a core part of AUD, but does not assess the same construct (in this case route of administration). Alcohol is consumed orally (T. Cicero (1980), but vapor inhalation is rarely (if ever) the route of administration.

1.4 Alcohol and inflammation

Using appropriate animal models to guide our hypotheses, we can further test pharmacological agents that reduce the harm of chronic alcohol consumption. Here, lets discuss recent evidence showing inflammation is an important pathway in response to alcohol, in the risk for alcohol drinking and a promising area of research for AUD treatment. Starting broadly, inflammation can be thought of as triggered responses after tissue insult from physical causes or pathogens (Zhou et al., 2010). The acute responses can be a result of various soluble proteins working to tackle the damage.

Alcohol as discussed above, can lead to tissue damage, therefore, studying how alcohol affects cytokines and how cytokines can protect against, or repair tissue damage is crucial.

1.4.1 Overview of cytokines and chemokines

Cytokines are small proteins secreted by various cell types (lymphocytes, monocytes, leukocytes, neurons, glia) that act on the same cells they are produced by, nearby cells, or even distant cells (Zhang and An., 2007). Cytokines have plenty of redundancy when it comes to their activity, thus, the same phenotype can occur despite different patterns of activation or inhibition (Zhang and An., 2007). Cytokines play crucial roles in normal brain function and various disease processes. They engage in processes like synapse development, plasticity, and communication between brain cells. Dysregulation of cytokine activity is implicated in neurological and psychiatric disorders (Dantzer, 2018). Chemokines, which are chemotactic cytokines, are described as small-secreted proteins that are mostly involved in leukocyte activation and migration (Zhang and An., 2007). Chemokines in the brain have been shown to play roles in normal brain function as well as immune responses. They are involved in leukocyte migration to sites of inflammation but also in developmental processes, cellular communication, angiogenesis, survival apoptosis, neuroprotection, and various others (reviewed in depth by Hughes and Nibbs, 2018).

Pro-inflammatory cytokines and chemokines tend to be the first secreted proteins that are targeted at sites of injury, inflammation, infection, or exogenous invasion (Zhang and An., 2007). Some examples that fall under this umbrella include IL-1β and TNF-α (see **table 1** for further examples).

Anti-inflammatory cytokines and chemokines modulate the actions of pro-inflammatory cytokines to return the cell environment to a homeostatic state. Prominent

anti-inflammatory cytokines include IL-4, IL-13, and IL-10 (see **Table 1** for more examples).

Cytokines can also have both pro- and anti-inflammatory actions after secretion, such as IL-6, G-CSF, and IL-9. These cyto- and chemokines require specific conditions that lead to one effect versus the other. A brief example, IL-6, can lead to anti-inflammatory signalling such as the activation of the signal transducer and activator of transcription 3 (STAT3) pathway in membrane bound IL-6 receptors (IL-6R) in the intestine. Whereas the soluble form of IL-6R can lead to increased pro-inflammation and is often associated with chronic inflammatory disorders (Scheller et al., 2011).

Table 1: Cytokine names, abbreviations and type

Cytokine	Abbreviation and alternatives	Pro- or Anti-inflammatory
Interleukin – 1 alpha	IL-1α	Pro-inflammatory
Interleukin – 1 beta	IL-1β	Pro-inflammatory
Interleukin – 2	IL-2	Pro- and anti-inflammatory
Interleukin – 3	IL-3	Pro- and anti-inflammatory
Interleukin – 4	IL-4	Anti-inflammatory
Interleukin – 5	IL-5	Pro-inflammatory
Interleukin – 6	IL-6	Pro- and anti-inflammatory
Interleukin – 9	IL-9	Pro- and anti-inflammatory
Interleukin – 10	IL-10	Anti-inflammatory
Interleukin – 12p40	IL-12p40	Pro-inflammatory
Interleukin – 12p70	IL-12p70	Pro-inflammatory
Interleukin – 13	IL-13	Anti-inflammatory

Interleukin – 17A	IL-17A	Pro-inflammatory
Eotaxin (CCL11 chemokine)		Pro-inflammatory
Granulocyte colony-stimulating factor	G-CSF (CSF-3)	Pro- and anti-inflammatory
Granulocyte-macrophage colony-stimulating factor	GM-CSF (CSF2)	Pro- and anti-inflammatory
Interferon gamma	IFN-γ	Pro-inflammatory
Keratinocyte chemoattractant (C-X-C motif chemokine)	KC (CXCL1)	Pro-inflammatory
Monocyte chemoattractant protein -1	MCP-1 (CCL2)	Pro-inflammatory
Macrophage inflammatory protein 1 alpha	MIP-1α (CCL3)	Pro-inflammatory
Macrophage inflammatory protein 1 beta	MIP-1β (CCL4)	Pro-inflammatory
Regulated upon Activation, Normally T-Expressed, and Presumably Secreted	RANTES (CCL5)	Pro-inflammatory
Tumor necrosis factor alpha	TNF-α	Pro-inflammatory

1.4.2 Harmful effects of alcohol

Using tissue obtained from the New South Wales Tissue Resource Center in Australia, He and Crews (2008) characterized the postmortem inflammatory landscape of individuals with AUD. One method of measurement that the authors used to determine pro-inflammation, was measurement of the chemokine MCP-1. MCP-1 has been shown to be involved in various pathways, but for our purpose here, it is involved in the excessive production of pro-inflammatory cytokines such as IL-1β and TNF-α (He

and Crews., 2008). He and Crews (2008) showed that MCP-1 protein levels were higher in the ventral tegmental area (VTA), substantia nigra (SN), hippocampus, and amygdala from those determined to be heavy lifetime drinkers as compared to control brains. The authors showed increased microglia immunoreactivity in those same brains compared to control brains. In 2013, Crews et al., found a positive correlation between lifetime alcohol consumption and the immunoreactivity of high mobility group box 1 (HMGB1) in the orbitofrontal cortex (OFC), a brain region important for working memory, taste and odor reward, learning and reversal associations, and other motivated behaviors (see in depth review Rolls, 2004).

A complementary study in the same manuscript utilized the excessive intragastric (i.g.) chronic ethanol assay on B6 mice to measure Toll-like receptor (TLR) mRNA and protein expression. Crews et al., (2013) found an increase in expression of TLR 2, 3, and 4 after i.g. administration of 5 g/kg ethanol for 10 days. The increased expression they saw also correlated with the HMGB1 findings, suggesting that these signals related to pro-inflammation are upregulated together after chronic ethanol exposure (Crews et al., 2013).

There is extensive evidence for TLR4 and neuroimmune responses; in a systemic review, 26.3% (40/151) published original research articles involving AUD and neuroimmune responses mentioned TLR4 in the results section (James et al., 2024). For in depth review of TLR and AUD results see Bachtell et al., 2015, Kong et al., 2023 and Meredith et al., 2021. Lifetime alcohol consumption has been shown to be correlated with TLR2, TLR3, and TLR4 signaling in moderate drinkers compared to controls (Crews et al., 2013). Crews et al., (2013) showed that chronic intragastric ethanol (5 g/kg) administration in B6 mice led to an increase in TLR2, TLR3 and TLR4 immunoreactive cells in the entorhinal cortex, a region involved in various memory types such as temporal association (Morrissey and Takehara-Nishiuchi, 2014). Others showed the

involvement of Toll like receptor 4 (TLR4) activation leading to increased levels of the transcription factor nuclear factor kappa light chain enhancer of activated B cells (NF-κB), after ethanol exposure in cultured cerebral cortex astrocytes from 21-day old rats (Blanco et al., 2005). In contrast, another study demonstrated that striatum from TLR4-KO mice had no differences in cytokine expression compared to wild type mice after chronic ethanol consumption (Pascual et al., 2015). The studies mentioned above provide insight into ethanol's wide-ranging effects on different cytokines.

One study of non-cirrhotic alcohol-dependent participants showed a correlation between amount of alcohol consumed and pro-inflammatory cytokines (notably in peripheral blood) found a positive correlation between mRNA levels of pro-inflammatory cytokines IL-1β and IL-8 and amount of alcohol consumed (Leclercq et al., 2014).

Importantly, inflammation and alcohol have been shown to be positively correlated depending on the duration of ethanol exposure, the brain region of interest, and which sex of animal you look at. As an example, one study tested rats using three different ethanol vapor exposure times and measured the mRNA levels of IL-6, chemokine ligand-2 (CCL2, also known as MCP-1) and TNF-α in the ventromedial prefrontal cortex (vmPFC), basolateral amygdala (BLA), NAc, and ventral tegmental area (VTA), and compared male and females (Baxter-Potter et al., 2017). Baxter-Potter et al., (2017) found that the BLA had a significant increase in TNF-α mRNA in rats who received acute ethanol vapor exposure. For the chronic ethanol vapor exposure (6 weeks), the authors found that mRNA levels of IL-6 and CCL2 were significantly increased in the vmPFC. In the NAc, chronic ethanol exposure increased levels of IL-6, CCL2 and TNF-α (compared to air vapor controls; Baxter-Potter et al., 2017). Interestingly, in the VTA and the BLA, chronic ethanol exposure led to an increase in CCL2 mRNA only in male rats compared to male controls (Baxter-Potter et al., 2017). This study provides evidence of the importance of experimental design, whereby brain region, time of mRNA extraction and

analysis, and sex of the animal can provide different outcomes under the same overarching hypothesis. So far, we've discussed the effects that alcohol can have on different cytokine and chemokine pathways. Scientists can take advantage of this relationship to develop and test therapeutics, further increasing the available options for patients who may not react well to our current medications.

1.4.3 Treatments targeting inflammation

Pharmacologically targeting immune signaling as a therapeutic treatment of AUD and its genetic risk factors has been successfully implemented (Cherry et al., 2014, Gibson et al., 2006, Ozburn et al., 2020). One genetic risk factor of AUD, as described earlier (see section 1.1) is binge drinking. We use DID, a high throughput behavioral assay, and mice bred to drink to intoxication to test novel compounds for efficacy in reducing excessive drinking. The Ozburn lab has employed a rigorous approach for testing whether compounds targeting immune signaling could reduce binge-like drinking in HDID-1 mice (Ferguson et al., 2018, Grigsby et al., 2020, and Ozburn et al., 2020). There have been ~30 compounds (with known effects on immune signaling in peripheral tissues) tested in HDID-1 mice, and about half of them were found to reduce binge-like drinking. Further research into the compounds that reduced drinking revealed they all played a role in increasing anti-inflammatory cytokine signaling (such as IL-10, Grigsby et al., 2020, Ozburn et al., 2020). Compounds that did not reduce drinking also had something in common, many reduced pro-inflammatory cytokines in the periphery (Ferguson et al., 2018, Grigsby et al., 2020). Alongside these findings, previous work has shown the importance of phosphodiesterase (PDE) signaling on anti-inflammation, as well as reducing ethanol consumption (Bell, et al., 2015, Blednov et al., 2014, 2018, 2022, 2023, Liu et al., 2017, Ozburn et al., 2020)

PDEs are diverse in their structure, specificity, tissue and cell distribution (Francis et al., 2011). They are described as a family of enzymes that generally

metabolize cyclic adenosine monophosphate (cAMP, Page and Spina, 2011). PDE4 inhibition has been discussed for its effects in inflammatory diseases as well, whereby, PDE4A, PDE4B, PDE4C, and PDE4D involved medications have been shown to help with the treatment of asthma, psoriasis, and even chronic inflammatory bowel disease (Jin et al., 2012).

One non-specific PDE inhibitor of interest, with anti-inflammatory effects, ibudilast, has been shown to reduce ethanol intake in animal models and in humans (Bell et al., 2015, Grodin et al., 2021, 2022). Ibudilast, a neuroimmune modulator and PDE inhibitor has garnered attention for its effects on ethanol intake (Gibson et al., 2006). Ibudilast was shown to have selective inhibitory effects for PDE3A, PDE4, PDE10, and PDE11 (Gibson et al., 2006). Using three different rodent models of dependence, ibudilast decreased ethanol intake during a two-hour, two-bottle choice assay in selectively bred alcohol-preferring rats and high-alcohol drinking rats, and in mice who went through CIE (Bell et al., 2015). Recently, a clinical trial involving non-treatment-seeking individuals (N = 52) who met the criteria for mild to severe AUD symptoms were given ibudilast (20 mg days 1-2, 50 mg days 3-14) or placebo for two weeks, and asked to report their feelings of drinking, craving and their mood daily in an online diary (Grodin et al., 2021). Individuals who received ibudilast had a significant reduction in heavy drinking days as compared to those who received placebo (Grodin et al., 2021). A follow-up analysis of the same randomized clinical trial tested the effects of ibudilast on peripheral and central inflammatory markers such as IL-6, IL-10, and TNF-α (Grodin et al., 2022). Grodin et al., (2022) found that patients who received ibudilast had lower inflammatory metabolite levels compared to placebo controls. Magnetic resonance spectroscopy revealed this finding in the superior frontal white matter (SFWM) as measured by choline levels. The authors chose choline as a proxy for inflammation due to its involvement in glial activation or cell membrane injury (Grodin et al., 2022). A more

recent randomized clinical trial assessing the effects of ibudilast for the treatment of AUD and peripheral inflammation found different results. Ray et al., (2025) analysed a phase 2, 12 week, double masked, placebo-controlled randomized clinical trial of ibudilast (50 mg twice daily). This clinical trial assessed adults who were seeking treatment for moderate or severe AUD *DSM-5.* The primary outcome measurements that the clinical trial assessed include heavy drinking days (defined as > 4 for women, > 5 for men), drinks per day, drinks per drinking day, and percentage of days abstinent. They also measured plasma levels of TNF-α, IL-6, IL-8, IL-10 and IFN-γ. Ray et al., (2025) found no significant support for the efficacy of ibudilast on heavy drinking days, or other measurements compared to controls. They also did not observe any significant effects of ibudilast on their cytokines of interest obtained from peripheral blood. It is important to acknowledge the treatment seeking criteria used in this study compared to the previously described trial from Grodin et al., (2022). Ray et al., (2025) enrolled people seeking treatment, whereas Grodin et al., (2022) enrolled non-treatment seeking individuals. Despite these differences, further evidence is required to determine the efficacy of ibudilast across different stages of AUD. Ibudilast is not the only PDE inhibitor that has been evaluated in a clinical setting. Another promising drug, apremilast, has a wealth of evidence supporting its harm reduction potential of AUD.

Apremilast, a PDE4 inhibitor, also reduces ethanol consumption in several rodent models. One study that compared the effects of various PDE inhibitors on two bottle choice ethanol drinking and the DID (Blednov et al., 2014). This study showed male C57BL/6J mice had a reduction in their ethanol intake and preference by four different PDE4 inhibitors, rolipram (1 mg/kg), mesopram (5 mg/kg), piclamilast (1 mg/kg), and CDP850 (10 and 25 mg/kg). Blednov et al., (2014) also reported a decrease in the DID ethanol intake by mesopram (5 mg/kg), piclamilast (1 mg/kg) and CDP840 (25 mg/kg). Rolipram also reduced ethanol intake, but only when the higher dosage (5 mg/kg) was

used (Blednov et al., 2014). In 2018, Blednov et al., found that a single injection of apremilast was able to decrease ethanol intake and preference in a dose dependent manner during a continuous two-bottle choice assay (0, 5, 15, 30, 50 mg/kg). The authors found this reduction in both male and female C57BL/6J mice. Notably, ethanol clearance was not affected after pretreatment of apremilast (20 mg/kg, Blednov et al., 2018). More recently, apremilast was shown to work in preclinical models and in a clinical setting (Grigsby et al., 2023). Using both sexes from four different strains of mice, Grigsby et al., (2023) showed a decrease in ethanol consumption in a spectrum of ethanol consumption models. These models include early stage four day DID for binge-like drinking, a four week DID to assess chronic intake (and treatment), operant self-administration to test motivation for ethanol drinking, and a model of compulsive-like behaviour using a quinine adulterated ethanol assay, CIE, and CIE ± forced swim stress (FSS; Grigsby et al., 2023). To test the effects of apremilast in a clinical setting, Grigsby et al., (2023) performed a phase IIa double blind, placebo proof-of-concept (POC) study, testing the effects of apremilast in non-treatment seeking individuals who met criteria for moderate to severe AUD. Eleven days of apremilast treatment (90 mg/d) resulted in a significant reduction in number of drinks per day compared to placebo controls (Grigsby et al., 2023).

Although medications approved for AUD (disulfiram, acamprosate and naltrexone) do not target the immune system directly, it has been shown recently that they do appear to provide support against inflammation. Disulfiram pre-treatment recently was shown in vitro to decrease known pro-inflammatory cytokines such as TNF-α and MCP-1 (CCL2) secreted by macrophages stimulated by lipopolysaccharide (LPS, Chen et al., 2024). A review of naltrexone goes over extensive evidence for its potential treatment of immune related diseases (Li et al., 2018). In a mouse model of cerebral ischemia,

acamprosate (400 mg/kg) was found to provide protective effects as well as improve recovery as measured by neuronal density (Doeppner et al., 2015).

We have briefly described a small portion of research on alcohol and inflammation that has occurred in the past few decades, which are reviewed in-depth elsewhere (see Crews and Sakar., 2015, Erickson et al., 2019, Grantham et a., 2023, Lacagnina et al., 2017, Mayfield and Harris., 2017, Meredith et al., 2021, Moura et al., 2022). One take away from the reviews mentioned above, alcohol is altering cytokines directly and indirectly and thus targeting them for new therapeutic avenues could provide novel treatments.

1.5 Thesis aims

1.5.1 Experimental methodology used

A wealth of pre-clinical and human literature supports a significant role of the NAc in all stages of AUD (Koob and Volkow, 2010; 2014). We used inbred High Drinking in the Dark line-1 (iHDID-1) mice that continue to exhibit the drinking to intoxication phenotype of their selectively bred founders (High Drinking in the Dark; HDID). (i)HDID-1 mice reliably drink to intoxication (achieving BECs > 80 mg%) in a Drinking-in-the-Dark (DID) assay, have been shown to work to gain access to ethanol in operant self-administration assays, are genetically distinct from other strains of mice, and represent a unique genotype for drug screening (Crabbe et al., 2009, 2014, Barkley-Levenson & Crabbe 2014, Cherry et al., 2014, Sachin et al., 2012, Jensen et al., 2021). Many of the compounds that reduce drinking in other commonly used strains (e.g., C57BL/6J) do not reduce drinking in (i)HDID-1 mice, and similarly fail to reduce drinking in humans (Savarese et al., 2022, Crabbe et al., 2017), suggesting that (i)HDID-1 mice may provide a more clinically relevant model to test for therapeutic treatments (Crabbe et al., 2014). These mice reliably reach intoxicating BECs > 80mg % (equivalent to 80

mg/dL), which sets them up as a unique model of genetic risk for binge-like ethanol drinking (Barkley-Levenson & Crabbe., 2014). These mice were derived from HDID-1 mice and exhibit similar phenotypes as their non-inbred counterparts (Crabbe et al., 2014). Using iHDID-1 mice and the DID behavioral assay, we determined the effects of alcohol on cytokine levels and whether apremilast ameliorated those effects.

1.5.2 Research questions and objectives:

The hypothesis tested in this study was that early (four days of exposure) and initial (one day of exposure) binge-like drinking in iHDID-1 mice will lead to alterations in the balance of pro- and anti-inflammatory cytokines and apremilast would ameliorate the effects of ethanol on cytokine expression. To achieve this, we first tested the effects of ethanol and apremilast (40 mg/kg) on cytokine levels in iHDID-1 mice after a four day DID behavioral assay. To elucidate the effects of initial exposure, we tested the effects of ethanol (and apremilast) on cytokine protein levels after a single two-hour limited access exposure period. We hypothesized that iHDID-1 mice who consume ethanol have increased pro-inflammatory cytokine protein levels after a four day DID, and this same increase may also be seen in the initial one-time exposure to ethanol. We hypothesized that apremilast (40 mg/kg) decreases the expression of pro-inflammatory cytokines through its proposed anti-inflammatory actions. Finally, we hypothesized that females would have greater pro-inflammatory cytokine levels.

Chapter 2. Methodology

2.1 Animals

Experiments 1 and 2 used adult male and female iHDID-1 (S26.G F30 and S26.GF30) mice, aged 8-12 weeks. HDID-1 mice were inbred starting with selection 26, creating genetically identical mice. Mice were bred and maintained in the Veterans Affairs Portland Health Care System Veterinary Medical Unit, on a reverse 12 hour / 12 hour / light cycle, with lights off at 7:30am pacific standard time (PST). Experimental rooms were maintained at a temperature of 21 ± 1 °C. Purina 5LOD chow (PMI Nutrition International, Brentwood, Missouri, USA) was available *ad libitum*. Mice were housed in standard polycarbonate cages with stainless steel wire tops on Bed-o'cobs® bedding (The Andersons, Inc, Maumee, Ohio, USA), and habituated to single housing and sipper tubes 7 days prior to the experiment. All procedures were approved by the local Institutional Animal Care and Use Committee and were conducted in accordance with NIH Guidelines for the Care and Use of Laboratory Animals.

2.2 Experiment 1: Measuring effects of early-stage binge-like ethanol drinking (with and without apremilast treatment) on NAc cytokine levels

Male and female iHDID-1 mice intake levels were assessed during the Drinking-in-the-Dark (DID) limited access assay, using 20% ethanol (ethanol, v/v) or tap water for 2 hours, across a four-day period. Each day, home-cage water bottles were replaced with a 20% ethanol (or tap water) sipper tube, 3 hours into their dark cycle for 2 hours. On the 4th and final day, mice received an i.p. injection of apremilast (40 mg/kg or vehicle) 30-60 min prior to DID (3 x 2 factors: ethanol/water, apremilast/vehicle groups, male/female). Immediately after DID, mice who drank ethanol underwent peri-orbital blood collection (20uL) to measure BECs using gas chromatography (Model 6890N, Agilent Technologies, Palo Alto, California, USA), as described by Finn et al., 2007. Mice

who drank water were handled similarly but no blood sample was collected. Mice were then deeply anesthetized using a ketamine/xylazine cocktail (200 mg/kg & 20 mg/kg mg/mL, respectively) for intracardial perfusion with ice cold phosphate buffered saline (PBS) to remove circulating monocytes prior to brain collection. Tissue collection and processing are discussed below.

2.3 Experiment 2: Measuring effects of initial stage binge-like Ethanol drinking (with and without apremilast treatment) on NAc cytokine levels

To test whether the initial binge-drinking exposure was sufficient to alter cytokine protein levels, one day of DID (two hours) was carried out with apremilast (40 mg/kg) or vehicle administration (n = 7-9 per factor: ethanol/water, apremilast/vehicle groups, male/female). Home-cage water bottles were replaced with a 20% ethanol (or tap water) sipper tube, 3 hours into their dark cycle for 2 hours. Mice (iHDID-1, age: 8-11 weeks, S.26 GF34) received an i.p. injection of apremilast (40 mg/kg or vehicle) 30-60 min prior to the start of the DID. Immediately after the 2 hour DID , mice who drank ethanol underwent peri-orbital blood collection (20uL) to measure BECs using gas chromatography (Model 6890N, Agilent Technologies, Palo Alto, California, USA), as described by Finn et al., 2007. Mice who drank water were handled similarly but no blood sample was collected. Mice were then deeply anesthetized using a ketamine/xylazine cocktail (200 mg/kg & 20 mg/kg mg/mL respectively) for intracardial perfusion with ice cold PBS to remove circulating monocytes prior to brain collection. Tissue collection and processing are discussed below.

2.4 Drugs

Ethanol (200 proof, Decon Labs, King of Prussia, Pennsylvania, USA) was dissolved in tap water to a 20% ethanol solution (v/v, in tap water) for all experiments. The PDE4 inhibitor, apremilast (Toronto Research Chemicals, Ontario, Canada) was

prepared fresh on the day of administration in a Tween-80 (1.75% v/v in sterile saline) suspension.

2.5 BEC

Peri-orbital sinus blood samples (20 µL) were immediately obtained after the end of the DID using 20 µL disposable soda-lime glass microcapillary pipets (Kimble Glass, Millville, New Jersey, cat# 71900-20). Mice were scruffed and held against a stable surface, one of their eyes were bulged outward and the microcapillary pipet was inserted at the medial canthus at a 45-degree angle, aiming for the back of the orbital sinus, The blood samples were diluted into vials (Agilent Technologies, Santa Clara, California, cat# 18034882) containing 500 µL of BEC matrix and immediately crimped. The BEC matrix was prepared before the end of the DID by adding 29.9 µL of n-propanol (n-PrOH) to a 100mL volumetric flask and adding MilliQ water until the 100mL mark. To minimize n-PrOH evaporation, all vials were covered with plastic wrap. The samples were stored at -20° C until analysis. To determine BEC concentrations, we used ambient headspace sampling gas chromatography (Finn et al., 2007).

2.6 Tissue collection, processing, and protein quantification

To analyze the cytokine protein levels in the NAc, after intra-cardial perfusion, we obtained tissue samples using mouse steel coronal 1mm brain matrix (Ted Pella, Redding, California, cat# 15067) and a 1mm Miltex™ Biopsy punch with plunger (Ted Pella, Redding, California, cat# 15110-10) to take approximately four punches, flash froze them in liquid nitrogen in microcentrifuge tubes (2mL EZ Micro Test Tubes, Bio-Rad Laboratories, Hercules, California, USA, cat# 12021148) and stored them at -80C. We thawed our tissue samples on ice before adding 1x RIPA buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA, cat# 89900) containing cOmplete™ Protease Inhibitor Cocktail (MilliporeSigma, Burlington, Massachusetts, USA, cat# 11697498001).

The tissue was homogenized using a motorized pestle. The cell suspension was sonicated using an ultrasonic processor probe (Sonics & Materials Inc. Newton, Connecticut, cat# VCX130 PB; 25 Hz, 10 seconds on, 10 seconds off for 1:30min) to lyse the cells. Sample lysates were then centrifuged at 4 ºC for four minutes at 6000 x g. Supernatant was collected and the detergent compatible (DC) Protein Assay Kit II (Bio-Rad Laboratories, Hercules, California, USA, cat# 500-0112) was employed to quantify protein concentrations for subsequent cytokine and chemokine analysis.

2.7 Cytokine and Chemokine quantification

To test whether cytokine and chemokine protein levels were altered in our experiments, we used a multiplexing magnetic bead approach to measure a panel of 23 cytokines and chemokines (Bio-Plex™ Pro Mouse Cytokine 23-plex panel, Bio-Rad Laboratories, Hercules, California, USA) (Lot# 64521678 & 64565935) using the Bio-Plex 200 suspension array system (Luminex Bio-Plex™ 200 system, Bio-Rad). The Bio-Plex is a high-throughput fluidics device that is based on three main components. First, fluorescent labeled magnetic beads are used to bind to the molecules of interest. Second, a flow cytometer will measure biochemical reactions that occur on the beads by using two lasers and associated optics. Third, a digital processor then measures the signal of fluorescence. Bio-Rad offers 200 different fluorescently labeled beads that will react with specific reactants. Those reactants can specifically recognize different molecules of interest. To achieve this, they use a sandwich method to bind an antibody to the cytokine or chemokine of interest, and another to bind the beads. The target of interest concentration is determined using an eight-point standard curve after measuring its reaction with the bead fluorescence. According to the software guide for the bio-plex 200, a five-parameter logistic (5PL) equation was used to generate a standard curve. This means that 5 parameters were used to “fit the curve”,

- a = estimated response at infinite concentration

- b = slope of the tangent at midpoint
- c = midrange concentration or midpoint
- d = estimated response at zero concentration
- g = asymmetry factor.

This 5PL equation expands on the four-parameter logistic (4PL) by adding the asymmetry factor, which better models immunoassay data (Gottschalk and Dunn, 2005).

$$y = d + \frac{(a-d)}{[1+(x/c)^b]^g}$$

2.8 Statistical analysis

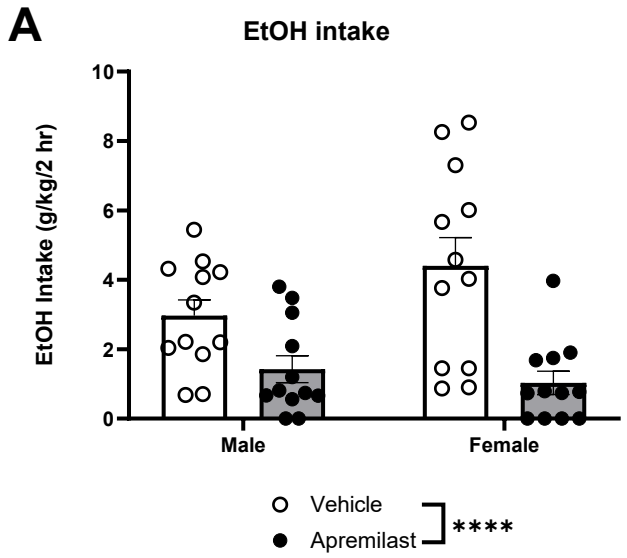
The group mean ± standard error of the mean (SEM) for intake data, BECs, and cytokine protein levels are shown with individual data points superimposed. Intake and BEC data were analyzed using a two-way analysis of variance (ANOVA; treatment x sex) and individual cytokine/chemokine protein levels were analyzed by three-way ANOVA (fluid x treatment x sex). Analyses were performed and graphs were prepared using GraphPad Prism v.10 (GraphPad Software, San Diego, CA, USA), with significance set at an alpha value of 0.05.

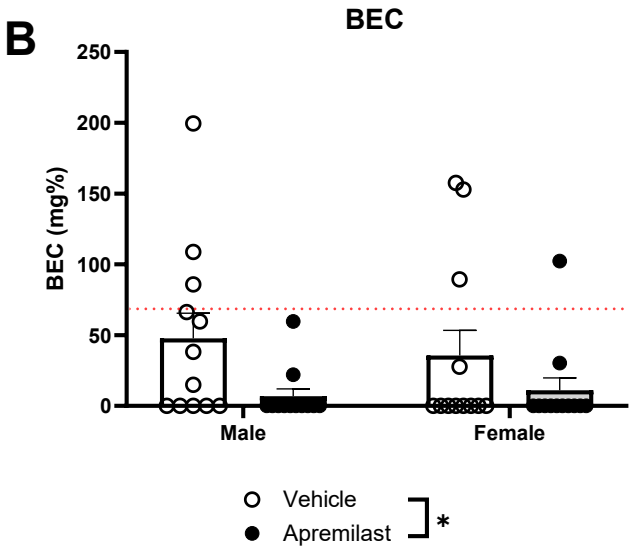
Outlier tests were run using R software and the > 2 standard deviations method, and we found no significant differences whether outliers were removed or not, therefore we chose to include them in the analysis. Values were excluded from analysis if they did not meet the following criteria: 1) an observed leak at the end of DID when measuring intake, 2) any cytokine protein value in which a mouse had replicate values with > 50% coefficient of variation (CV), 3) tissue samples that did not have enough measurable protein after cell lysis, and 4) tissue samples stored in 2mL microcentrifuge tubes at -80º C whose animal identity stickers fell off.

Chapter 3. Results

3.1 Early stage limited access fluid intake is decreased by apremilast.

We set out to test whether iHDID-1 mice drinking behavior could be ameliorated by Apremilast (40 mg/kg), and whether there was any significant effect of fluid type or sex. The mean (± SEM) and individual data points superimposed are presented in **Fig. 1.** Two-way ANOVA (treatment x sex) revealed mice who received 40 mg/kg (i.p) of apremilast during the fourth day of DID, had reduced 20% ethanol intake (**Fig. 1A**, main effect of treatment, $F(1, 44) = 20.52$, $p < 0.0001$). Two-way ANOVA (treatment x sex) analysis revealed that apremilast significantly reduced BECs compared to controls (**Fig. 1B**, main effect of treatment, $F (1, 44) = 5.879$, $p < 0.05$)). The red dashed line in **Fig. 1B** represents 80mg%, the NIAAA defined intoxication level. It is important to note that many of the animals had no measurable BECs but were still included in the analysis. These values are in line with previously reported BECs of iHDID-1 mice (Jensen et al., 2021) for a 2 hour DID. Two-way ANOVA (treatment x sex) analysis revealed that apremilast significantly reduced water intake as well (**Fig. 1C**, main effect of treatment, $F (1, 44) = 9.278$, $p = 0.0039$)). As **Fig. 1C** shows, there are ~10 animals who did not drink any water after apremilast treatment. This was reported in B6 mice with high doses of apremilast (30-50 mg/kg) reducing total fluid intake, which the authors suggest could be a result of sedation (Blednov et al., 2018). We did not observe any significant main effects of sex or interactions of treatment x sex. This is not the first study to show apremilast reducing alcohol intake in mice (Blednov et al., 2014, 2018a, 2018b, 2020, 2022, Crabbe et al., 2020, Grigsby et al., 2020, 2023, Ozburn et al., 2020). This study expands on previous research by implementing cytokine measurements alongside our intake data in iHDID-1 mice.


A
EtOH intake
EtOH Intake (g/kg/2 hr)
10
8
6
4
2
0
Male
Female
Vehicle
Apremilast
****


B
BEC
BEC (mg%)
250
200
150
100
50
0
Male
Female
Vehicle
Apremilast
*

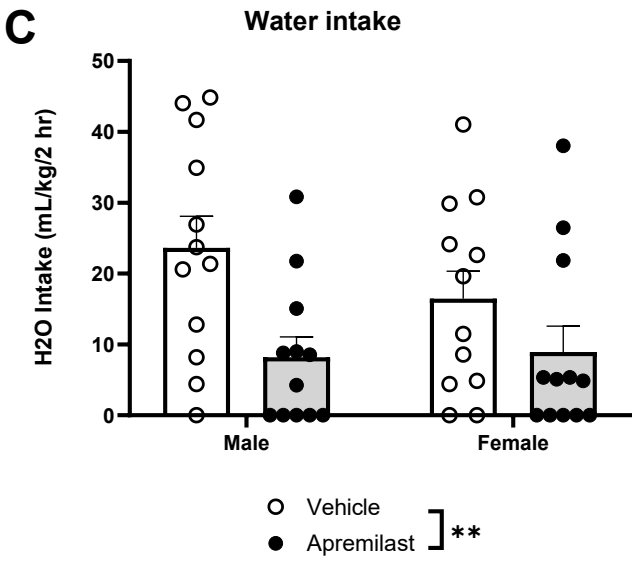

C
Water intake
H2O Intake (mL/kg/2 hr)
50
40
30
20
10
0
Male
Female
Vehicle
Apremilast
**

Fig. 1: Early stage limited access fluid intake is decreased by apremilast (40 mg/kg).

Apremilast reduced binge-like ethanol intake, BECs and water intake in iHDID-1 mice during the early-stage 4 Day DID. **(A)** Ethanol intake (g/kg/2hr) on Day 4($p < 0.0001$). **(B)** Blood ethanol levels (mg %), were significantly reduced ($p < 0.05$). **(C)** Water intake (mL/kg/2hr) was decreased on Day 4, ($p < 0.01$). Intake and BEC are presented as the mean ± SEM with individual data points superimposed. Red dashed line in (**B)** represents the defined level of intoxication = 80 mg%. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

3.2 Effects of early-stage binge-like drinking (with and without apremilast treatment) on the balance of anti- and pro-inflammatory cytokines and chemokines

To test whether ethanol altered the balance of pro- and anti-inflammatory cytokine protein levels in the NAc of iHDID-1 mice, we determined protein concentrations of 23 different cytokines and chemokines using the Bio-Rad Multiplex cytokine 23-plex kit. Separate three-way ANOVAs (fluid x treatment x sex) for each cytokine/chemokine was carried out. Three-way ANOVA (fluid x treatment x sex) analysis revealed 12/23 cytokines were found to have significant main effects of fluid, with ethanol drinking increasing the levels of these cytokines (**Fig. 2A-L**).Three-way ANOVA analysis revealed a significant increase in G-CSF protein levels (**Fig. 2G** main effect of fluid, $F(1, 19) = 5.9$, $p = 0.0255$, main effect of treatment, $F(1, 19) = 4.5$, $p = 0.0476$) but we did not observe a significant main effect of sex or any significant interactions. Three-way ANOVA analysis of KC revealed significantly higher protein levels in iHDID-1 mice who received ethanol (**Fig. 2I**, main effect of fluid, $F(1, 38) = 9.0$, $p = 0.0047$). We observed a significant fluid x treatment x sex interaction ($F(1, 38) = 4.4$, $p = 0.0432$) for the protein levels of KC in iHDID-1 mice. For an in-depth look at the statistics, see Refer to **Table 3** for the descriptive statistics (minimum, 25th percentile, median, 75th percentile, maximum, range, standard deviation, standard error of the mean, lower 95% confidence interval and higher 95% confidence interval) of all cytokines in the 4 day DID. 115 out of 1081 protein measurements were removed for not meeting our criteria mentioned in section **2.8.**

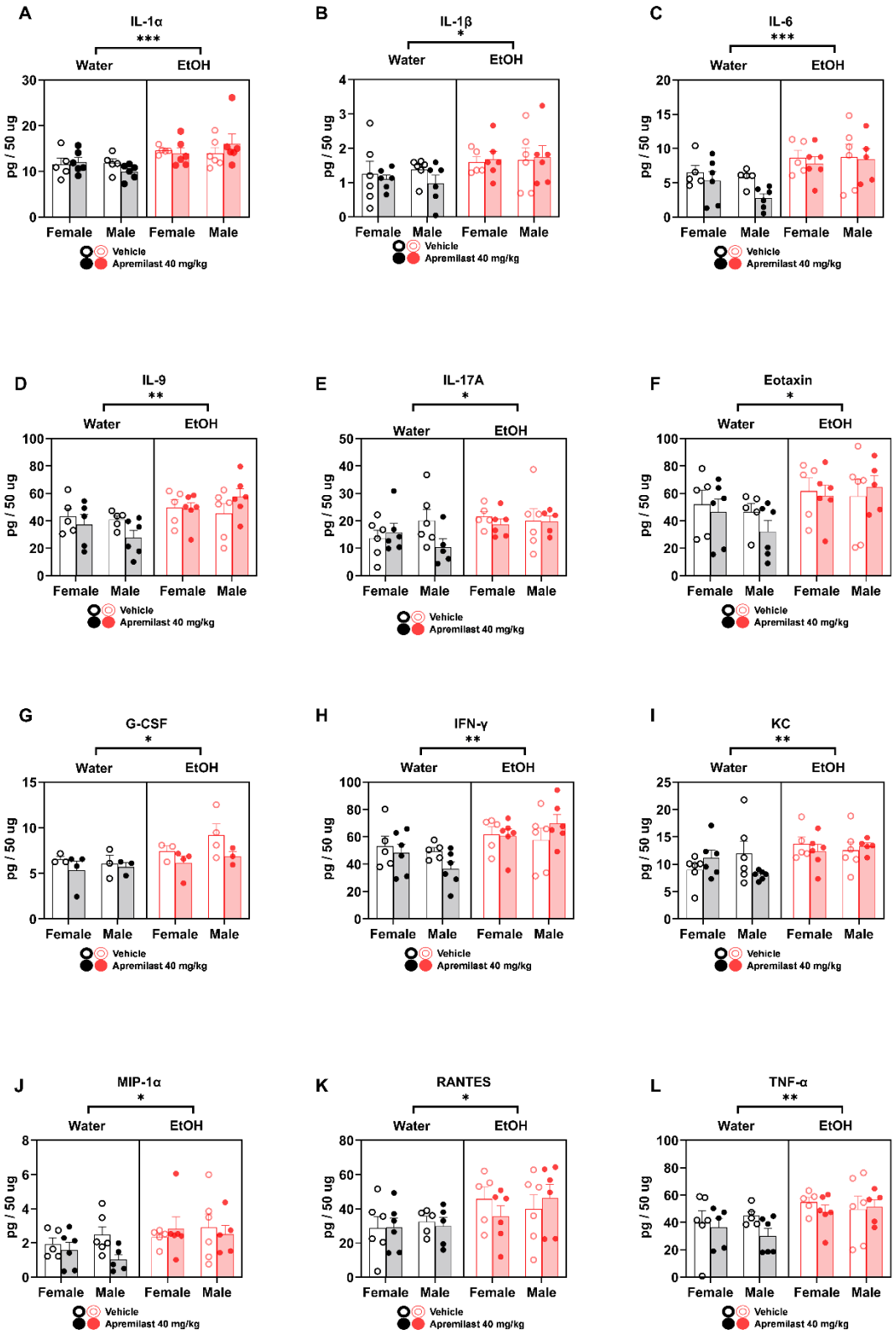

A
IL-1α
***
Water
EtOH
pg / 50 ug
Female
Male
Female
Male
Vehicle
Apremilast 40 mg/kg
B
IL-1β
*
C
IL-6
***
D
IL-9
**
E
IL-17A
*
F
Eotaxin
*
G
G-CSF
*
H
IFN-γ
**
I
KC
**
J
MIP-1α
*
K
RANTES
*
L
TNF-α
**

Fig 2: Alcohol increases pro-inflammatory cytokines/chemokines after early-stage binge-like drinking in iHDID-1 mice.

Four days of binge-like ethanol drinking increased IL-1α (2A; $p < 0.001$), IL-1β (2B; $p < 0.05$), IL-6 (2C; $p < 0.001$), IL-9 (2D; $p < 0.01$), IL-17A (2E; $p < 0.05$), Eotaxin (2F; $p < 0.05$), G-CSF [2G; $p < 0.05$, and treatment ($p < 0.05$)], IFN-γ (2H; $p < 0.01$), KC [2F; $p < 0.01$, and a fluid x treatment x sex interaction ($p < 0.05$)], MIP-1α (2J; $p < 0.05$), RANTES (2K; $p < 0.05$), and TNFα (2L; $p < 0.01$). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. We did not observe any effects of apremilast on ethanol intake.

Cytokine	Sex	Fluid	Treatment	Sex x Fluid
IL-1α	F(1, 37) = 0.0081	F(1, 37) = 14	F(1, 37) = 0.00042	F(1, 37) = 0.86
	P=0.9287	P=0.0006	P=0.9837	P=0.3610
IL-1β	F(1, 39) = 0.031	F(1, 39) = 6.8	F(1, 39) = 0.27	F(1, 39) = 0.027
	P=0.8607	P=0.0130	P=0.6072	P=0.8711
IL-2	F(1, 37) = 0.29	F(1, 37) = 3.3	F(1, 37) = 0.34	F(1, 37) = 0.043
	P=0.5943	P=0.0755	P=0.5619	P=0.8368
IL-3	F(1, 36) = 0.14	F(1, 36) = 1.8	F(1, 36) = 2.4	F(1, 36) = 0.0035
	P=0.7123	P=0.1906	P=0.1339	P=0.9531
IL-4	F(1, 35) = 0.10	F(1, 35) = 1.1	F(1, 35) = 1.1	F(1, 35) = 0.010
	P=0.7502	P=0.3042	P=0.3049	P=0.9193
IL-5	F(1, 24) = 0.0013	F(1, 24) = 4.1	F(1, 24) = 0.053	F(1, 24) = 0.011
	P=0.9712	P=0.0552	P=0.8205	P=0.9166
IL-6	F(1, 36) = 0.47	F(1, 36) = 15	F(1, 36) = 2.4	F(1, 36) = 1.3
	P=0.4957	P=0.0005	P=0.1278	P=0.2547
IL-9	F(1, 36) = 0.17	F(1, 36) = 10	F(1, 36) = 0.25	F(1, 36) = 1.0
	P=0.6847	P=0.0032	P=0.6206	P=0.3212
IL-10	F(1, 37) = 0.024	F(1, 37) = 1.8	F(1, 37) = 0.99	F(1, 37) = 0.12
	P=0.8778	P=0.1847	P=0.3261	P=0.7324
IL-12p40	F(1, 26) = 0.68	F(1, 26) = 1.1	F(1, 26) = 0.053	F(1, 26) = 0.35
	P=0.4175	P=0.3066	P=0.8196	P=0.5608
IL-12p70	F(1, 38) = 0.046	F(1, 38) = 3.0	F(1, 38) = 1.8	F(1, 38) = 0.056
	P=0.8316	P=0.0937	P=0.1869	P=0.8139
IL-13	F(1, 36) = 0.00068	F(1, 36) = 1.1	F(1, 36) = 0.12	F(1, 36) = 0.0051
	P=0.9794	P=0.3107	P=0.7338	P=0.9437
IL-17A	F(1, 37) = 0.0042	F(1, 37) = 5.1	F(1, 37) = 1.4	F(1, 37) = 0.028
	P=0.9488	P=0.0302	P=0.2400	P=0.8678
Eotaxin	F(1, 36) = 0.43	F(1, 36) = 6.2	F(1, 36) = 0.39	F(1, 36) = 0.71
	P=0.5150	P=0.0175	P=0.5367	P=0.4037
G-CSF	F(1, 19) = 0.92	F(1, 19) = 5.9	F(1, 19) = 4.5	F(1, 19) = 1.2
	P=0.3503	P=0.0255	P=0.0476	P=0.2941
GM-CSF	F(1, 32) = 0.19	F(1, 32) = 0.59	F(1, 32) = 0.0043	F(1, 32) = 0.023
	P=0.6694	P=0.4498	P=0.9482	P=0.8799
IFN-γ	F(1, 37) = 0.31	F(1, 37) = 12	F(1, 37) = 0.16	F(1, 37) = 1.3
	P=0.5780	P=0.0011	P=0.6947	P=0.2604
KC	F(1, 38) = 0.0050	F(1, 38) = 9.0	F(1, 38) = 0.37	F(1, 38) = 3.4e-005
	P=0.9441	P=0.0047	P=0.5465	P=0.9953
MCP-1	F(1, 30) = 0.54	F(1, 30) = 0.0018	F(1, 30) = 0.64	F(1, 30) = 1.0
	P=0.4671	P=0.9662	P=0.4283	P=0.3237
MIP-1α	F(1, 36) = 0.016	F(1, 36) = 5.5	F(1, 36) = 1.3	F(1, 36) = 0.030
	P=0.9007	P=0.0251	P=0.2673	P=0.8633
MIP-1β	F(1, 27) = 0.98	F(1, 27) = 1.3	F(1, 27) = 0.47	F(1, 27) = 2.5
	P=0.3307	P=0.2730	P=0.4983	P=0.1279
RANTES	F(1, 37) = 0.27	F(1, 37) = 6.4	F(1, 37) = 0.095	F(1, 37) = 0.0013
	P=0.6096	P=0.0155	P=0.7591	P=0.9718
TNF-α	F(1, 36) = 0.023	F(1, 36) = 7.8	F(1, 36) = 1.6	F(1, 36) = 0.0011
	P=0.8814	P=0.0084	P=0.2138	P=0.9743

Cytokine	Sex x Treatment	Fluid x Treatment	Sex x Fluid x Treatment
IL-1α	F(1, 37) = 0.017	F(1, 37) = 0.60	F(1, 37) = 2.1
	P=0.8968	P=0.4435	P=0.1541
IL-1β	F(1, 39) = 0.13	F(1, 39) = 0.95	F(1, 39) = 0.12
	P=0.7255	P=0.3364	P=0.7282
IL-2	F(1, 37) = 0.62	F(1, 37) = 0.34	F(1, 37) = 0.43
	P=0.4359	P=0.5654	P=0.5161
IL-3	F(1, 36) = 0.68	F(1, 36) = 1.6	F(1, 36) = 0.44
	P=0.4166	P=0.2090	P=0.5128
IL-4	F(1, 35) = 0.022	F(1, 35) = 0.30	F(1, 35) = 0.0033
	P=0.8832	P=0.5881	P=0.9543
IL-5	F(1, 24) = 0.89	F(1, 24) = 0.069	F(1, 24) = 0.11
	P=0.3548	P=0.7957	P=0.7480
IL-6	F(1, 36) = 0.14	F(1, 36) = 0.75	F(1, 36) = 0.46
	P=0.7087	P=0.3907	P=0.5019
IL-9	F(1, 36) = 0.13	F(1, 36) = 3.3	F(1, 36) = 1.5
	P=0.7207	P=0.0798	P=0.2245
IL-10	F(1, 37) = 0.16	F(1, 37) = 0.33	F(1, 37) = 0.12
	P=0.6915	P=0.5678	P=0.7275
IL-12p40	F(1, 26) = 0.61	F(1, 26) = 0.62	F(1, 26) = 0.11
	P=0.4433	P=0.4394	P=0.7404
IL-12p70	F(1, 38) = 0.69	F(1, 38) = 0.57	F(1, 38) = 1.1
	P=0.4112	P=0.4539	P=0.3001
IL-13	F(1, 36) = 0.48	F(1, 36) = 0.010	F(1, 36) = 0.033
	P=0.4948	P=0.9194	P=0.8577
IL-17A	F(1, 37) = 1.1	F(1, 37) = 0.27	F(1, 37) = 2.6
	P=0.3031	P=0.6047	P=0.1134
Eotaxin	F(1, 36) = 0.0032	F(1, 36) = 0.78	F(1, 36) = 0.49
	P=0.9554	P=0.3824	P=0.4893
G-CSF	F(1, 19) = 0.0046	F(1, 19) = 0.70	F(1, 19) = 0.59
	P=0.9467	P=0.4141	P=0.4504
GM-CSF	F(1, 32) = 0.44	F(1, 32) = 0.020	F(1, 32) = 0.025
	P=0.5137	P=0.8878	P=0.8747
IFN-γ	F(1, 37) = 0.088	F(1, 37) = 2.6	F(1, 37) = 1.5
	P=0.7689	P=0.1164	P=0.2289
KC	F(1, 38) = 1.1	F(1, 38) = 0.096	F(1, 38) = 4.4
	P=0.3070	P=0.7590	P=0.0432
MCP-1	F(1, 30) = 1.7	F(1, 30) = 0.090	F(1, 30) = 0.065
	P=0.2025	P=0.7660	P=0.8001
MIP-1α	F(1, 36) = 1.7	F(1, 36) = 1.6	F(1, 36) = 0.024
	P=0.1999	P=0.2144	P=0.8766
MIP-1β	F(1, 27) = 0.012	F(1, 27) = 0.0097	F(1, 27) = 0.020
	P=0.9138	P=0.9222	P=0.8881
RANTES	F(1, 37) = 0.52	F(1, 37) = 0.0069	F(1, 37) = 1.1
	P=0.4738	P=0.9344	P=0.3021
TNF-α	F(1, 36) = 0.012	F(1, 36) = 0.46	F(1, 36) = 1.1
	P=0.9125	P=0.5035	P=0.2956

Table 2: Three-way ANOVA analysis table for 4-day DID. Represented here is the f value and the p value for each cytokine and each factor. The table was split in two for ease of readability.

Cytokine	Number of values	Minimum	25% Percentile	Median	75% Percentile	Maximum	Range	Mean	Std. Deviation	Std. Error of Mean	Lower 95% CI of mean	Upper 95% CI of mean
IL-1a	45	7.2	11	12	15	26	19	13	3.3	0.49	12	14
IL-1b	47	0.04	0.97	1.4	1.8	3.2	3.2	1.4	0.65	0.095	1.2	1.6
IL-2	45	10	25	39	50	66	56	39	14	2.1	35	44
IL-3	44	0.18	1.7	2.2	2.9	5.4	5.3	2.3	1.2	0.17	1.9	2.6
IL-4	43	0.17	1.8	2.3	3.1	10	10	2.9	2.4	0.36	2.1	3.6
IL-5	32	0.19	1.3	2.4	3.3	5	4.8	2.2	1.2	0.22	1.8	2.7
IL-6	44	0.51	4.2	6.3	8.9	15	14	6.7	3.3	0.5	5.7	7.7
IL-9	44	10	33	46	55	80	69	44	15	2.3	39	48
IL-10	45	5.7	21	59	78	114	108	54	32	4.8	44	63
IL-12(p40)	34	0.52	6.8	11	15	32	32	13	8.3	1.4	10	16
IL-12(p70)	46	33	83	97	113	235	203	101	36	5.3	91	112
IL-13	44	6.7	21	77	92	124	118	61	38	5.7	49	73
IL-17A	45	3	13	17	22	39	36	18	7.7	1.1	15	20
Eotaxin	44	8.9	30	56	69	94	86	52	22	3.4	45	59
G-CSF	27	2.5	6.1	6.6	7.6	13	10	6.7	1.8	0.35	6	7.4
GM-CSF	40	4.1	18	22	26	43	39	23	7.5	1.2	20	25
IFN-g	45	17	42	54	66	94	77	55	17	2.5	50	60
KC	46	3.8	8.6	11	13	22	18	11	3.6	0.53	10	12
MCP-1	38	7.7	59	103	115	181	173	92	39	6.3	79	105
MIP-1a	44	0.34	1.3	2.1	2.7	6.1	5.7	2.2	1.3	0.2	1.8	2.6
MIP-1b	35	12	43	55	67	106	94	55	20	3.3	48	62
RANTES	45	3.5	23	36	50	64	61	36	16	2.4	31	41
TNF-a	44	0.84	38	45	57	76	75	44	16	2.4	39	49

Table 3: **Descriptive statistics of 4 day DID cytokine protein expression.**

3.3 Effects of apremilast on initial binge-like drinking

To test whether an initial experience of binge-like drinking, with or without apremilast treatment, altered the balance of cytokines, we implemented a one day DID (n = 16 / sex / treatment) behavioral assay and processed the NAc brain tissue of iHDID-1 mice as described in experiment 1. Two-way ANOVA (treatment x sex) analysis revealed that apremilast significantly decreased ethanol intake in iHDID-1 mice (**Fig. 3A**, main effect of treatment, $F (1, 28) = 7.9$, $p = 0.0091$) but no sex or treatment by sex interactions were observed. We did not observe any significant main effects of treatment, sex or a treatment by sex interaction on BECs (**Fig. 3B**). Important to note that only 5 animals reached intoxication, as defined by the red dashed line (80 mg%), which is not unusual. HDID-1 mice were shown to drink very little total ethanol on day 1 of a DID, which could explain why we see low intake and BECs (Barkley-Levenson and Crabbe, 2012). Two-way ANOVA (treatment x sex) analysis revealed that apremilast significantly reduced water intake in iHDID-1 mice **(Fig. 3C**, main effect of treatment, $F (1, 29) = 9.2$, $p = 0.0050$) but no main effect of sex or a treatment by sex interaction was observed. Like the 4 day DID, a decrease in water intake by apremilast could be due to the high dose we chose to use for these studies (40 mg/kg).

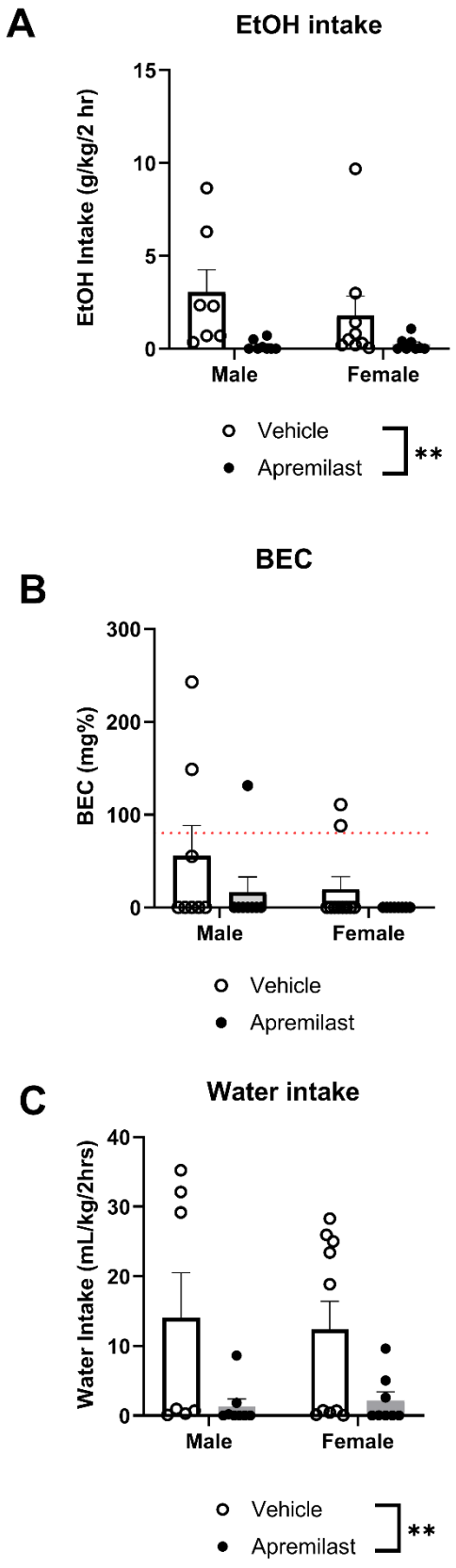


Fig. 3: Apremilast decreases initial fluid intake in iHDID-1 mice. Apremilast reduced binge-like ethanol intake, BECs and water intake in iHDID-1 mice during the initial 1 day DID. **(A)** Ethanol intake was significantly reduced during the 1 day DID in iHDID-1 mice ($p < 0.01$). **(B)** There were no statistically significant findings of apremilast on BECs. **(C)** Apremilast significantly decreased water intake during the 1 day DID in iHDID-1 mice ($p < 0.01$). Red dashed line in (**B**) represents the defined level of intoxication = 80 mg%. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

3.4 Initial binge-like alcohol drinking (with or without apremilast treatment) did not alter cytokine protein levels.

To test the effects of initial limited access binge-like drinking after apremilast treatment in iHDID-1 mice, we asked whether 1 day of DID would be sufficient to alter the balance of NAc cytokine protein levels. Three-way ANOVA (fluid x treatment x sex) did not reveal any significant effects of fluid or treatment. We did observe a significant main effect of sex in 18 out of 23 cytokines, but we are underpowered and did not *a priori* set out to test these effects (see **Table 4** for three-way ANOVA results). Refer to **Table 5** for the descriptive statistics (minimum, 25th percentile, median, 75th percentile, maximum, range, standard deviation, standard error of the mean, lower 95% confidence interval and higher 95% confidence interval) of all cytokines in the 1 day DID. The sex effects we observed after 1 day compared to no sex effects observed after 4 days could be due to baseline immune differences between male and female iHDID-1 mice which becomes negligible when compared to the effects of ethanol on cytokines. 71 out of 1334 protein measurements were excluded from analysis for not meeting the criteria mentioned in section **2.8**.

Cytokine	Sex	Fluid	Treatment	Sex x Fluid
IL-1α	F(1, 50) = 3.1	F(1, 50) = 0.0064	F(1, 50) = 0.26	F(1, 50) = 0.12
	P=0.0835	P=0.9368	P=0.6104	P=0.7307
IL-1β	F(1, 50) = 7.7	F(1, 50) = 0.63	F(1, 50) = 0.11	F(1, 50) = 1.5
	P=0.0078	P=0.4311	P=0.7393	P=0.2303
IL-2	F(1, 50) = 5.1	F(1, 50) = 0.0053	F(1, 50) = 0.00058	F(1, 50) = 2.2
	P=0.0290	P=0.9425	P=0.9809	P=0.1424
IL-3	F(1, 50) = 7.2	F(1, 50) = 1.2	F(1, 50) = 0.49	F(1, 50) = 0.92
	P=0.0100	P=0.2821	P=0.4891	P=0.3415
IL-4	F(1, 34) = 3.6	F(1, 34) = 2.5	F(1, 34) = 1.3	F(1, 34) = 0.31
	P=0.0676	P=0.1210	P=0.2645	P=0.5798
IL-5	F(1, 47) = 6.5	F(1, 47) = 0.22	F(1, 47) = 1.0	F(1, 47) = 2.1
	P=0.0144	P=0.6383	P=0.3161	P=0.1576
IL-6	F(1, 49) = 9.9	F(1, 49) = 0.65	F(1, 49) = 0.00014	F(1, 49) = 1.5
	P=0.0028	P=0.4235	P=0.9907	P=0.2213
IL-9	F(1, 49) = 5.0	F(1, 49) = 0.88	F(1, 49) = 0.039	F(1, 49) = 2.5
	P=0.0296	P=0.3521	P=0.8448	P=0.1225
IL-10	F(1, 50) = 7.7	F(1, 50) = 0.25	F(1, 50) = 1.4	F(1, 50) = 1.2
	P=0.0077	P=0.6165	P=0.2454	P=0.2789
IL-12p40	F(1, 50) = 3.4	F(1, 50) = 0.93	F(1, 50) = 1.2	F(1, 50) = 0.77
	P=0.0728	P=0.3407	P=0.2772	P=0.3859
IL-12p70	F(1, 48) = 9.0	F(1, 48) = 0.38	F(1, 48) = 0.58	F(1, 48) = 2.3
	P=0.0042	P=0.5401	P=0.4514	P=0.1356
IL-13	F(1, 50) = 6.5	F(1, 50) = 0.29	F(1, 50) = 0.16	F(1, 50) = 2.4
	P=0.0136	P=0.5935	P=0.6873	P=0.1268
IL-17A	F(1, 50) = 8.2	F(1, 50) = 0.61	F(1, 50) = 0.16	F(1, 50) = 2.1
	P=0.0062	P=0.4384	P=0.6867	P=0.1506
Eotaxin	F(1, 50) = 5.3	F(1, 50) = 0.69	F(1, 50) = 0.010	F(1, 50) = 3.3
	P=0.0254	P=0.4089	P=0.9206	P=0.0765
G-CSF	F(1, 50) = 14	F(1, 50) = 0.15	F(1, 50) = 0.34	F(1, 50) = 1.0
	P=0.0006	P=0.6967	P=0.5629	P=0.3150
GM-CSF	F(1, 47) = 0.77	F(1, 47) = 1.8	F(1, 47) = 0.23	F(1, 47) = 0.81
	P=0.3836	P=0.1875	P=0.6328	P=0.3730
IFN-γ	F(1, 50) = 8.0	F(1, 50) = 0.22	F(1, 50) = 0.86	F(1, 50) = 1.0
	P=0.0067	P=0.6385	P=0.3590	P=0.3222
KC	F(1, 50) = 11	F(1, 50) = 0.44	F(1, 50) = 0.53	F(1, 50) = 2.4
	P=0.0015	P=0.5115	P=0.4708	P=0.1248
MCP-1	F(1, 50) = 6.6	F(1, 50) = 0.0029	F(1, 50) = 0.00096	F(1, 50) = 3.4
	P=0.0133	P=0.9569	P=0.9754	P=0.0706
MIP-1α	F(1, 28) = 9.8	F(1, 28) = 0.016	F(1, 28) = 1.4	F(1, 28) = 0.11
	P=0.0041	P=0.9010	P=0.2522	P=0.7449
MIP-1β	F(1, 27) = 13	F(1, 27) = 0.0060	F(1, 27) = 1.6	F(1, 27) = 0.026
	P=0.0015	P=0.9386	P=0.2166	P=0.8733
RANTES	F(1, 50) = 7.6	F(1, 50) = 0.38	F(1, 50) = 0.85	F(1, 50) = 1.7
	P=0.0081	P=0.5396	P=0.3596	P=0.1922
TNF-α	F(1, 50) = 2.8	F(1, 50) = 0.50	F(1, 50) = 0.19	F(1, 50) = 0.019
	P=0.1019	P=0.4838	P=0.6668	P=0.8905

Cytokine	Sex x Treatment	Fluid x Treatment	Sex x Fluid x Treatment
IL-1α	F(1, 50) = 0.62	F(1, 50) = 1.1	F(1, 50) = 0.14
	P=0.4336	P=0.3062	P=0.7092
IL-1β	F(1, 50) = 0.58	F(1, 50) = 0.60	F(1, 50) = 0.15
	P=0.4498	P=0.4426	P=0.7045
IL-2	F(1, 50) = 1.4	F(1, 50) = 0.69	F(1, 50) = 1.3
	P=0.2501	P=0.4115	P=0.2611
IL-3	F(1, 50) = 0.47	F(1, 50) = 0.79	F(1, 50) = 0.20
	P=0.4968	P=0.3786	P=0.6570
IL-4	F(1, 34) = 0.049	F(1, 34) = 0.33	F(1, 34) = 0.083
	P=0.8265	P=0.5669	P=0.7751
IL-5	F(1, 47) = 1.4	F(1, 47) = 0.053	F(1, 47) = 0.017
	P=0.2480	P=0.8183	P=0.8979
IL-6	F(1, 49) = 0.077	F(1, 49) = 1.2	F(1, 49) = 0.96
	P=0.7826	P=0.2806	P=0.3311
IL-9	F(1, 49) = 0.016	F(1, 49) = 0.097	F(1, 49) = 0.012
	P=0.8999	P=0.7573	P=0.9128
IL-10	F(1, 50) = 0.78	F(1, 50) = 0.98	F(1, 50) = 0.64
	P=0.3822	P=0.3266	P=0.4262
IL-12p40	F(1, 50) = 0.046	F(1, 50) = 1.1	F(1, 50) = 0.10
	P=0.8317	P=0.3015	P=0.7475
IL-12p70	F(1, 48) = 0.17	F(1, 48) = 0.29	F(1, 48) = 0.045
	P=0.6800	P=0.5920	P=0.8333
IL-13	F(1, 50) = 3.8	F(1, 50) = 0.35	F(1, 50) = 1.3
	P=0.0562	P=0.5547	P=0.2548
IL-17A	F(1, 50) = 0.88	F(1, 50) = 0.52	F(1, 50) = 0.11
	P=0.3526	P=0.4758	P=0.7436
Eotaxin	F(1, 50) = 0.56	F(1, 50) = 5.9e-006	F(1, 50) = 0.0066
	P=0.4563	P=0.9981	P=0.9354
G-CSF	F(1, 50) = 2.4	F(1, 50) = 0.85	F(1, 50) = 0.17
	P=0.1239	P=0.3597	P=0.6846
GM-CSF	F(1, 47) = 0.0079	F(1, 47) = 0.38	F(1, 47) = 0.17
	P=0.9294	P=0.5405	P=0.6836
IFN-γ	F(1, 50) = 0.19	F(1, 50) = 0.39	F(1, 50) = 0.00086
	P=0.6652	P=0.5372	P=0.9768
KC	F(1, 50) = 1.4	F(1, 50) = 0.39	F(1, 50) = 0.19
	P=0.2348	P=0.5377	P=0.6619
MCP-1	F(1, 50) = 1.5	F(1, 50) = 0.018	F(1, 50) = 0.47
	P=0.2258	P=0.8941	P=0.4956
MIP-1α	F(1, 28) = 2.1	F(1, 28) = 0.87	F(1, 28) = 0.25
	P=0.1560	P=0.3588	P=0.6240
MIP-1β	F(1, 27) = 2.5	F(1, 27) = 1.1	F(1, 27) = 0.097
	P=0.1288	P=0.2960	P=0.7581
RANTES	F(1, 50) = 3.2	F(1, 50) = 0.21	F(1, 50) = 0.68
	P=0.0786	P=0.6473	P=0.4129
TNF-α	F(1, 50) = 0.00052	F(1, 50) = 1.4	F(1, 50) = 0.19
	P=0.9818	P=0.2411	P=0.6637

Table 4: Three-way ANOVA analysis table for 1 day DID. Represented here is the f value and the p value for each cytokine and each factor. The table was split in two for ease of readability.

Cytokine	Number of values	Minimum	25% Percentile	Median	75% Percentile	Maximum	Range	Mean	Std. Deviation	Std. Error of Mean	Lower 95% CI of mean	Upper 95% CI of mean
IL-1a	59	3.17	6.8	9.32	12.69	28.41	25.24	11.04	5.782	0.7527	9.531	12.54
IL-1b	59	2.94	8.58	10.52	13.94	48.59	45.65	14.21	10.37	1.35	11.51	16.91
IL-2	59	81.66	164.3	206.1	286.6	591.2	509.5	241.9	113.5	14.77	212.3	271.4
IL-3	59	2.5	5.79	8.28	10.52	30.05	27.55	9.544	5.945	0.774	7.995	11.09
IL-4	43	1.55	4.85	7.03	9.96	24.64	23.09	8.155	5.351	0.816	6.508	9.802
IL-5	56	2.05	4.025	6.155	11.51	24.72	22.67	8.153	5.803	0.7754	6.599	9.707
IL-6	58	0.47	6.075	8.35	10.96	32.94	32.47	9.993	6.717	0.882	8.226	11.76
IL-9	58	5.23	17.31	24.18	34.56	90.73	85.5	28.78	19.42	2.549	23.68	33.89
IL-10	59	19.78	60.43	79.23	115	203.9	184.1	88.19	42.72	5.561	77.06	99.32
IL-12(p40)	59	16.48	40.11	57.64	86.59	181.8	165.3	67.37	37.71	4.91	57.55	77.2
IL-12(p70)	57	35.87	71.28	91.97	154.5	392.2	356.4	127.4	85.7	11.35	104.7	150.1
IL-13	59	63.41	192	327.6	587	1076	1012	395.1	248.8	32.39	330.2	459.9
IL-17A	59	7.34	11.58	18.52	30.59	89.92	82.58	25.06	20.37	2.651	19.76	30.37
Eotaxin	59	17.91	33.48	48.67	71.89	210.2	192.3	60.56	40.84	5.317	49.91	71.2
G-CSF	58	6.71	20.18	37.18	56.41	157.9	151.1	46.49	36.54	4.798	36.88	56.1
GM-CSF	56	0	13.6	26.3	35.48	73.18	73.18	27.44	17.79	2.377	22.68	32.2
IFN-g	59	5.87	24.6	31.57	48.92	132.7	126.8	41.12	26.96	3.51	34.09	48.15
KC	59	8.16	16.98	23.4	34.91	97.86	89.7	29.55	20.25	2.636	24.28	34.83
MCP-1	59	100.4	148.5	248.9	431.6	960.5	860.1	309.6	187.2	24.37	260.8	358.4
MIP-1a	36	1.68	2.78	3.975	7.503	24.67	22.99	6.289	5.76	0.9601	4.34	8.238
MIP-1b	35	36.46	45.7	63.34	135.8	255	218.5	93.8	62	10.48	72.5	115.1
RANTES	58	49.16	82.31	111.9	179.1	359.5	310.4	138.8	71.31	9.363	120	157.5
TNF-a	59	21.79	63.15	82.04	158.8	366.2	344.5	114.9	72.19	9.398	96.06	133.7

Table 5: Descriptive statistics of 1 day DID cytokine protein expression.

Cytokine	4 day DID	1 day DID
IL-1a	2	0
IL-1b	0	0
IL-2	2	0
IL-3	3	0
IL-4	4	16
IL-5	15	3
IL-6	3	1
IL-9	3	1
IL-10	2	0
IL-12(p40)	13	0
IL-12(p70)	1	2
IL-13	3	0
IL-17A	2	0
Eotaxin	3	0
G-CSF	20	0
GM-CSF	7	3
IFN-g	2	0
KC	1	0
MCP-1	9	0
MIP-1a	3	22
MIP-1b	12	23
RANTES	2	0
TNF-a	3	0
	115	71

Table 6 Number of protein values excluded from each cytokine per study.

Chapter 4. Discussion

4.1 Overall findings

Here, we examined the effects of ethanol and apremilast on the balance of cytokines after early (four days) and initial (one day) exposure to Drinking-in-the-Dark (DID) in iHDID-1 male and female mice. We observed a significant decrease of ethanol intake in early and initial exposures to DID after apremilast treatment. We also observed a decrease in water intake after apremilast treatment during the early and initial 1 day DID assay. A decrease in water and ethanol intake suggests that the effects of apremilast are not specific to ethanol. This was in line with previous findings from the lab and others (Blednov et al., 2014, 2018a, 2018b, 2020, 2022, Crabbe et al., 2020, Grigsby et al., 2020, 2023, Ozburn et al., 2020). We hypothesized a decrease in pro-inflammatory cytokines after apremilast treatment, but no such finding was observed. Female mice will generally consume more ethanol than their male counterparts (Crabbe et al., 2009, Rath et al., 2020, Rhodes et al., 2005, Sneddon et al., 2019), which led us to hypothesize a stronger effect of ethanol on cytokine protein expression in female mice compared to their male ethanol counterparts. Only KC had a significant interaction of fluid x treatment x sex, although post hoc multiple comparison testing did not reveal any significant findings. We expected higher levels of pro-inflammatory cytokines in the ethanol vehicle groups, but this finding was not exclusive to vehicle treated mice who drank ethanol. Instead, we observed the highest mean pro-inflammatory cytokine levels across both ethanol exposed treatment (apremilast and vehicle) groups. During the 4-day DID, the 12 significant cytokines we observed were all associated with pro-inflammatory signaling, although IL-3, IL-6 and IL-9 have been associated with anti-inflammatory signaling as well (Goswami and Kaplan, 2011, Podolska et al., 2024, Sheller et al., 2011).

Recently, Monnig et al., (2025) tested the effects of acute alcohol-induced changes in LPS and alcohol on a panel of 8 pro-inflammatory cytokines and chemokines. Here, the authors used placebo, low-dose or moderate dose alcohol, in patients determined to be light or heavy drinkers, at baseline and every hour for four hours. Monnig et al., (2025) found cytokine and chemokine effects to be dependent on the dose, time or drinking history. For example, they saw no effects of dose or drinker group on IL-10, but did observe a decrease in baseline expression after four hours. To contrast, IL-6 was higher across conditions if participants were deemed light drinkers but saw suppression in participants deemed heavy drinkers (Monnig et al., 2025). Monnig et al., (2025) described above and our experiments in **Chapter 3** help to fill a gap in the literature, demonstrating acute alcohol is sufficient to alter cytokines and chemokines, but the direction of effects depend on other variables such as past drinking history, time after alcohol exposure and model organism of choice. The Monnig et al., (2025) study and our initial 1 day DID have different results, although this could be the two-hour difference after alcohol when the blood samples were collected, the history of alcohol drinking or just differences between human (Monnig et al., 2025) and rodent (**Chapter 3**) cytokine expression patterns. In our 1 day DID experiment, iHDID-1 mice are naïve at the start of testing, and tissue is collected at the end of the 2-hour DID, in contrast, IL-6 and MCP-1 show the largest expression changes from 2-4 hours in the Monnig (2025) paper. We did observe a similar finding as Monnig et al., (2025) in our 4 day DID, where a history of drinking (days 1-3) led to observed effects of ethanol on cytokines. As I will discuss below, the contradictory findings are not surprising but important to consider.

4.2 Modeling binge-like drinking in iHDID-1 mice.

Although iHDID-1 mice were bred to drink to intoxication, their amount of intake and BEC levels can still vary. While many of the vehicle treated ethanol drinking mice in the 4 day DID reached or exceeded intoxicating BECs, not many mice reached intoxicating BECs after the 1 day DID. This makes it a bit more difficult to compare the immune results across the two experiments. Because we were interested in volitional drinking, we did not control for equal dosing, where a bender (sometimes referred to as a gavage model) could have provided insight. For example, if all ethanol mice received the same amount of ethanol using an intragastric (i.g.) oral gavage, we would expect a more representative measure of the biological variance in cytokine levels resulting from controlled ethanol levels (see Crews et al., 2013) for 4 days or 1 day. Alongside this, iHDID-1 mice were all exposed to DID, but we did not capture the baseline cytokine expression levels of naïve mice who did not go through experimental conditions.

4.2.1 Sex differences in immune protein levels

Based on previous literature (Crabbe et al., 2009, Rath et al., 2020, Rhodes et al., 2005, Sneddon et al., 2019), we expected to see sex specific differences in ethanol intake during DID, where females drink more than males. We hypothesized that sex differences on ethanol intake could lead to sex specific effects in cytokine protein expression levels between male and female mice. Klein and Flanagan (2016) discuss why measuring sex specific effects are important for immune related studies. Klein and Flanagan (2016) discuss evidence for the epidemiological theory on why females were found to represent 80% of autoimmune diseases in humans (Invernizzi et al., 2009, Klein and Flanagan, 2016). A large proportion of immune related genes are located on the x chromosome, whereby females, who typically have two x chromosomes, will have increased chances of problematic mutations (Angum et al., 2020, Invernizzi et al., 2009).

Another explanation of sex specific immune responses in females, is the differences in mRNA and protein levels of estrogen receptors in female mice compared to male counterparts. Kovats (2015) reviews this theory citing the differences in estrogen receptor mRNA and protein expression to explain sex specific immune functions. Estrogen has been shown to have dose dependent immune functions, where lower doses are pro-inflammatory and higher doses are anti-inflammatory (Kovats, 2015). One explanation for this could be through the inhibition of NF- κB, a transcription factor important for inducing pro-inflammatory genes (Liu et al., 2017). Transcription activity is inhibited when the estrogen receptor alpha subunit combines with NF-κB subunits (Kovats, 2015). A review of estrogen and psoriasis also describes this pathway, citing NF-κB's involvement in the expression of pro-inflammatory cyto- and chemokines IL-1β, monocyte chemoattractant protein 1 (MCP-1) and KC (Adachi and Honda, 2022). Kovats (2015) describes a similar relationship of estrogen and chemokine MCP-1. As shown in **Table 1**, MCP-1 is a chemokine with known pro-inflammatory effects when suppressed by estrogen receptor alpha binding in place of the cAMP response element-binding protein (CREB)-binding protein (CBP, Kovats 2015). A systematic review of sex hormones and alcohol consumption provides conflicting results. Erol et al., (2019) found that estrogen and ethanol intake are positively correlated in most animal studies they reviewed, but there were conflicting studies that found a negative correlation or no effect. Interestingly, for the human studies that Erol et al., (2019) reviewed, estrogen plays more of a role in adult female social drinkers, when estradiol levels are sustained during the luteal phase, they reported increased intake.

The 1 day DID study reported main effects of sex on cytokine levels. The effect of sex on cytokine levels did not appear in the 4 day DID. To better understand why this might have occurred, as discussed above, there are sex differences in immune

signalling. Since we had various mice not drink much ethanol, perhaps our 1 day DID findings are capturing those baseline sex differences, which gets lost once mice drink ethanol for more than 1 day. There is no way to fully know without adding additional mice and measuring their cytokine levels before any ethanol exposure as a comparison. Another explanation could be using only 1 day of DID, rather than the well established 2 day protocol.

With the various sex differences discussed above, it remains crucial to study sex as a biological variable in immune related studies, otherwise we dismiss the most at-risk population for immune related disease states.

4.2.2 Diurnal rhythms in immune protein levels

Cytokine and chemokine measurements are complicated by the fact that tissue analysed at various times of day can have variable measurements (Nakao, 2014). One study analysed the diurnal expression patterns of the soluble IL-2 receptor and soluble TNF receptor subunits p55 and p75 in 22 healthy males involved in a sleep study (Haack et al., 2004). This study found that plasma levels of soluble TNF receptor subunits had significant diurnal variation, when measured over 24 hours, with peak z-scored levels from 22:00 – 06:00 hours (Haack et al., 2004). Another study found that human variant cytokines IL-6, TNF-α and IFN-γ levels peak in early morning evidenced by in vitro lipopolysaccharide (LPS) stimulation of whole blood plasma (Petrovsky et al., 1998). These diurnal variations are altered by inflammatory disease states as well. One study measured the circulating blood levels of IL-6 and TNF-α from patients with various rheumatic diseases (and healthy controls) and found that patients with rheumatoid arthritis had a significant decrease in mean serum levels during the day, when compared to early morning levels (10:30 vs 13:30), a classic example of diurnal rhythms (Arvidson et al., 1994). Of note, IL-6 serum levels were not detectable in healthy controls (Arvidson

et al., 1994) and TNF-α levels did not exhibit these same patterns. Another study measured blood circulating IL-2 levels from ten healthy volunteers and found that IL-2 levels significantly increased during the nighttime compared to the daytime levels (Lissoni et al., 1998).

Alcohol drinking disrupts expression of several circadian genes in various peripheral and brain tissues (Burgess et al., 2022, Davis et al., 2018, Grigsby et al., 2022, Miller et al., 2023, Ozburn et al., 2013, Parekh et al., 2015). Some of these circadian genes are transcription factors that regulate diurnal expression of thousands of genes, many of which are important for neurotransmission and neuroimmune signaling (Davis et al., 20218, Ozburn et al., 2015, Parekh et al., 2014). Diurnal rhythm effects on cytokine expression needs further investigation, however, until then, it remains important to document when samples of interest are taken. The experiments described in chapter 2 followed established DID timelines, starting three hours into the dark (active) cycle. Thus, a single timepoint may not have captured the extent to which alcohol and/or apremilast alters cytokine and/or chemokine levels.

4.2.3 Tissue specific cytokine production

Measures of cytokine protein expression are influenced not only by circadian rhythms, but also by tissue type and location. Zubaidi et al., (2015) used an enzyme linked immunosorbent assay (ELISA) assay to determine the different tissue protein concentrations in the skin, colon, muscle, and small intestine at 5 different timepoints (day 1, 3, 5, 7, and 14) after anastomosis surgery. Briefly, the authors found that IL-6 levels in the skin did not significantly differ across days, but in the small intestine, IL-6 levels had the lowest expression on day 1 and 3 after surgery and the highest expression on day 5 and 14 (97.8-105.3 pg/mL – 254.3-227.2 pg/mL respectively). It is important to note that brain tissue was not analysed in Zubaidi et al., (2015), which

warrant central cytokine kinetic time course studies. The experiments described in **Chapter 2** do not assess different types of tissue or kinetics, which suggests our results should not be extrapolated to other brain areas, and our experimental control group protein measurements only capture one timepoint of twenty-three different cytokines.

Typically, a clinical study involving cytokine and chemokine analysis, will often perform the measurements at the end of the study (De Jager et al., 2009). Scientists must consider the best method of long-term storage to allow for accurate measurements. A study led by De Jager et al., 2009 measured cytokine degradation obtained from the blood of four healthy volunteers, at baseline and then every year for 4 years after that. The cytokines that were measured were IL-1α, IL-1β, IL-2, IL-4 IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-18, TNF- α, CXCL8 and IFN-γ. It turns out that even one of our better methods of storage (-80 ºC freezers) can both increase and decrease the cytokine and chemokine measurements (De Jager et al., 2009). This study on long term cytokine degradation found that IL-13 and IL-17 degrade within a year after blood sample collection. De Jager et al., (2009) determined that IL-1α, IL-1β, IL-5, IL-6, and IL-10 are degraded up to 50% or less of baseline within 2-3 years. The study also found that IL-2, IL-4, and IL-12 were stable up to 3 years.

Long term storage conditions can have differing effects depending on the cytokine. Furthermore, the authors (De Jager et al., 2009) tested how the cytokines mentioned above are affected after thawing by comparing aliquot measurements. De Jager and colleagues found that IL-6 and IL-10 are stable after multiple freeze – thawing cycles. Interestingly, IL-4 and TNF-α levels can rise after one or more freeze-thaw cycles. Conversely, IL-13, IL-15, IL-17, IFN-γ, and CXCL8 dropped after 3 freeze-thaw cycles compared to baseline (De Jager et al., 2009). **Chapter 2** experiments limited the amount of freeze-thaw to 2 cycles (one for cell lysis and protein quantification and one for

running the Bioplex 200 immunoassay), and all samples were processed in duplicate within 6 months of storing at -80C.

Cytokines and chemokines are dynamic with differing half-lives. The half-life of a protein for our purposes is defined as the time it takes for the concentration of a protein to be reduced to 50%. The research on cytokine half-lives is sparse, however, Table 1 from Liu et al., 2021 summarizes some of the known examples from mice. Briefly, Liu et al., 2021 review various studies and obtained these limited results:

- IL-1β half-life = 21 minutes
- IL-6 half-life = 15.5 hours
- IL-8 half-life = 24 minutes
- IL-12 half-life = not available
- TNF-α half-life = 18.2 minutes
- IFN-γ half-life = not available
- IL-4 half-life = 20 minutes
- IL-10 half-life = not available
- IL-13 half-life = not available

While the cytokine half-life measurements were taken from different in vitro bodily fluids such as serum, plasma, saliva, tears or stool, the review critically points out the variance that cytokine expression could have over the course of minutes to hours. Lastly, an antibody recognition sequence for soluble cytokines may not detect different protein complex combinations, when many different combinations of soluble cytokines and binding proteins are possible (Liu et al., 2021). This is likely to be a more important consideration for immunohistochemistry in fixed tissues than in the lysates and bead-base immunoassays used in our studies. The limitations mentioned thus far are

important to consider, whilst recognizing that a single experiment cannot capture and consider all variables that influence protein expression. For **Chapter 2** experiments, we did not consider cytokine half-life or antibody recognition sequences, suggesting that we captured a single snapshot of a dynamic environment.

The animals used in the experiments for this thesis include male and female iHDID-1 mice, which are a unique inbred model maintained by the Ozburn lab. It has been shown that genetically different strains of mice can have assay dependent behaviors (Jensen et al., 2021, Rhodes et al., 2007). Without further experimental evidence, it is not clear whether these findings generalize to other strains of mice or species.

Without over-generalizing, the results of the studies described in **Chapter 3**are still important. We add additional evidence supporting apremilast as a method of harm reduction. Apremilast has been shown to reduce heavy drinking days in non-treatment seeking individuals as well as reducing motivation and binge-like drinking in iHDID-1 mice (Grigsby et al., 2023). Our study replicates the binge-like reductions of ethanol consumption, converging on evidence of apremilast as a treatment option for AUD or its risk factors. We did not find any significant effects of treatment or fluid on cytokines and chemokines after 1 day of DID, which could be explained by no previous ethanol history, or dissecting tissue samples from the NAc immediately after DID, rather than allowing for ethanol clearance or withdrawal symptoms. A review/meta-analysis on AUD and circulating cytokines came to a similar conclusion, where they reported the largest differences in cytokine concentrations came from patients actively drinking or in withdrawal, when compared to patients who were abstinent from alcohol and control (Adams et al., 2020).

Binge-like alcohol drinking is a genetic risk factor for AUD and with these insights, implementing harm reduction methods early on, could help prevent some of the negative consequences seen with chronic alcohol consumption. Regardless, pro-inflammatory immune signaling is a consequence of alcohol drinking, and there exists great therapeutic potential for compounds like apremilast and ibudilast.

Conclusion

We show a decrease in ethanol intake after one treatment of apremilast in iHDID-1 male and female mice. We show an increase of pro-inflammatory cytokines after as little as 4 days of ethanol binge-like limited access exposure. The experimental approach we chose was sufficient to answer our original hypotheses to test whether: 1) the effects of ethanol binge-like drinking alter the balance of cytokines in the NAc, 2) apremilast can ameliorate the effects of ethanol binge-like drinking on cytokine levels, and 3) an initial exposure to ethanol binge-like drinking can lead to changes in cytokine levels. Alongside these findings, we are left with many new research questions. Some of these questions include:

- What does naïve baseline cytokine expression look like in iHDID-1 mice?
- How important is the half-life of cytokines for protein expression?
- Does plasma and serum cytokine levels show similar patterns as our brain tissue lysate did?
- What does cytokine expression look like after a period of withdrawal from alcohol (12 hours, 24 hours, 1 week)?
- Can we use the cytokine expression levels we observe as biomarkers for drinking or treatment response?

Future directions to consider are vast. Although inbred mice provide phenotypic and genotypic stability, using a heterogeneous stock of mice would provide insight on generalizability, unfortunately, the vast majority don't drink to intoxication, so this is not a possibility. A logical next step could be replicating this study using the iHDID-2 mice, to determine the impact of drinking and apremilast in mice with a different genotype that exhibit the same phenotype. Future studies could measure levels of immune proteins in other brain areas , since we know that multiple brain regions are involved in addiction (Koob and Volkow 2016). The research described within this thesis adds to our limited understanding of pro- and anti-inflammatory protein levels after binge-like drinking, a risk factor for AUD. This basic research also provides converging evidence on the effectiveness of apremilast on binge-like drinking.

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