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Epigenetic Mechanisms Regulating the Functional Effects of Chronic Alcohol Exposure of Human Monocyte-derived Dendritic Cells

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

EPIGENETIC MECHANISMS REGULATING THE FUNCTIONAL EFFECTS OF CHRONIC ALCOHOL EXPOSURE OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS.

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL SCIENCES

by

Tiyash Parira

2018
To: Dean John A Rock  
Herbert Wertheim College of Medicine

This dissertation, written by Tiyash Parira, and entitled Epigenetic Mechanisms Regulating the Functional Effects of Chronic Alcohol Exposure of Human Monocyte-derived Dendritic Cells, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Date of Defense: November 6, 2018

The dissertation of Tiyash Parira is approved.

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And Senior Vice President of Medical Affairs

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Andrés G. Gil  
Vice President for Research and Economic Development  
And Dean of the University Graduate School

Florida International University, 2018
DEDICATION

I dedicate this dissertation to my family for their support and to my advisor Dr. Marisela Agudelo who helped me in all things great and small.
ACKNOWLEDGMENTS

I would like to thank my Advisor, Dr. Marisela Agudelo for guiding me and mentoring me through my doctoral studies. Her constant guidance and accessibility regarding academic and non-academic affairs have given me the support I needed as an international graduate student. Her ideas and criticism have helped my project grow to have a successful completion that it has achieved. She has been there to always give me a push in the right direction and make me a better scholar and researcher every day. I am proud to have her as my mentor and major professor.

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ABSTRACT OF THE DISSERTATION

EPIGENETIC MECHANISMS REGULATING THE FUNCTIONAL EFFECTS OF CHRONIC ALCOHOL EXPOSURE OF HUMAN MONOCYTE-DERIVED DENDRITIC CELLS.

by

Tiyash Parira

Florida International University, 2018

Miami, Florida

Professor Marisela Agudelo, Major Professor

The effects of alcohol abuse are multi-dimensional since alcohol is widely known to affect both the innate and adaptive immune systems. Recently, epigenetics has come into focus and has been implicated in many diseases as well as substance abuse disorders. Therefore, research efforts of understanding the epigenetic mechanisms underlying substance abuse effects including alcohol abuse have become more predominant.

In our laboratory, we have studied different epigenetic changes induced by alcohol exposure including regulation of histone deacetylases (HDACs), histone quantity, and histone modifications such as acetylation and deacetylation. We have observed differential effects of acute and chronic alcohol exposure in human monocyte-derived dendritic cells (MDDCs) wherein our laboratory previously found that HDACs were modulated in MDDCs treated acutely with alcohol in vitro, and in MDDCs from alcohol users. Our previous work has also demonstrated that alcohol consumption affects the dendritic cell function by modulating inflammatory markers and cannabinoid
receptors such as CB₂ and GPR55 through epigenetic modifications. For instance, chronic alcohol exposure upregulates histone (H) 4 acetylation at lysine 12 (H4k12ac) and acute alcohol effects on histone acetylation are associated with an increase in GPR55 expression.

The hypothesis of the study is that chronic alcohol modulates human MDDC function through epigenetic mechanisms. Therefore, the primary objective of this research project is to elucidate novel pathways involving histone post-translational modifications due to chronic alcohol exposure in human dendritic cells.

For this study, monocytes isolated from commercially available human buffy coats were differentiated into MDDCs, which were treated with chronic alcohol levels of 0.1 % (100mg/dL) and 0.2 % (200mg/dL) for 5 days in the presence or absence of the histone acetyltransferase inhibitor NU9056 (50nM) or the GPR55 antagonist CID16020046 (5µM). Results showed that chronic alcohol levels upregulated H4K12ac in MDDCs and this was associated with a concomitant increase in GPR55 gene and protein expression. Further, NU9056 and CID16020046 were able to reduce alcohol-induced inflammatory chemokine MCP-2 and reactive oxygen species production indicating that H4K12ac may be an inflammation and oxidative stress regulator. Additionally, NU9056 and CID16020046 could potentially reduce alcohol-induced inflammation and serve as potential therapeutic targets for alcohol use disorders.
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ABBREVIATIONS AND ACCRONYMS

%........................................................................................................Percent
4-MP..............................................................................................4-Methylpyrazole
ADH.............................................................................................Alcohol dehydrogenase
AF 647..............................................................................................Alexa Fluor 647
APC.................................................................................................Antigen Presenting Cell
ATF2...............................................................................................Activating transcription factor 2
AUDs..............................................................................................Alcohol Use Disorders
cAMP.............................................................................................Cyclic adenosine monophosphate
CB1..............................................................................................Cannabinoid Receptor 1
CB2..............................................................................................Cannabinoid Receptor 2
CD..................................................................................................Cluster of Differentiation
ChIP...............................................................................................Chromatin Immuno-precipitation
CID...............................................................................................CID16020046
CREB............................................................................................cAMP response element-binding protein
CYP2E1..........................................................................................Cytochrome P450 2E1
DAPI ............................................................................................4',6-diamidino-2-phenyindole
DC.................................................................................................Dendritic Cell
DCF-DA ......................................................................................2',7'-dichlorodihydrofluorescein diacetate
DNA ................................................................. Deoxyribo Nucleic Acid
DNMT ............................................................... DNA methyl Transferases
DUI ................................................................. Driving Under Influence
ELISA .......................................................... Enzyme Linked Immunosorbant Assay
ERK .............................................................. Extracellular Signal-Regulated Kinase
EtOH ............................................................. Ethanol or Alcohol
FAAH .......................................................... Fatty-acid amide hydrolase
FASD ............................................................. Fetal Alcohol Spectrum disorder
FITC ............................................................. Fluorescein Isothiocyanate
GABA .......................................................... Gamma-aminobutyric acid
GAPDH ......................................................... Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF ....................................................... Granulocyte-macrophage colony-stimulating factor
GPCR .......................................................... G Protein-Coupled Receptor
GPR55 .......................................................... G protein-coupled receptor 55
H3 ................................................................. Histone 3
H3K9 ........................................................... Histone 3 Lysine 9
H3K9ac ......................................................... Histone 3 Lysine 9 acetylation
H4 ................................................................. Histone 4
H4K12ac ..................................................... Histone 4 Lysine 12 acetylation
HAT .......................................................... Histone acetyl transferase

HDAC .......................................................... Histone deacetylase

HLA-DR .......................................................... Human Leukocyte Antigen – DR Isotype

ICAM-1 .......................................................... Intercellular Adhesion Molecule 1

Igf1 .......................................................... Insulin-like growth factor 1

IL .......................................................... Interleukin

LDH .......................................................... Lactate dehydrogenase

Lim2 .......................................................... Lens intrinsic membrane protein 2

LPS .......................................................... Lipopolysaccharide

MALDI-FT-ICR-MS ........................................... Matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry

MAP Kinase ................................................. Mitogen-activated protein kinase

MCP-2 .......................................................... Monocyte Chemotactic Protein-2

MDDCs .......................................................... Monocyte-derived dendritic cells

MFI .......................................................... Mean Fluorescent Intensity

MHC .......................................................... Major histocompatibility complex

MIP-2 .......................................................... Macrophage inflammatory protein-2

MS .......................................................... Mass Spectrometry

NFAT .......................................................... Nuclear factor of activated T-cells
NIAAA.................................National Institute on Alcohol Abuse and Alcoholism
nM..........................................................nano Molar
NMDA .............................................................N-methyl-D-aspartate
NSDUH........................................ National Survey on Drug Use and Health
PBMCs..................................................Peripheral blood mononuclear cells
PCR.............................................................Polymerase chain reaction
PLC..........................................................Phospho lipase C
PPAR..................................................Peroxisome proliferator-activated receptors
qRT-PCR....................................................Quantitative real time PCR
RASGRF2........................ Ras-specific guanine nucleotide-releasing factor 2
RhoA.......................................................Ras homolog gene family, member A
RNA..........................................................Ribonucleic acid
ROS..........................................................Reactive oxygen species
SD..............................................................Standard deviation
SDS PAGE..........................Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
SEM..........................................................Standard error of mean
siRNA..................................................small interfering RNA
TAI..........................................................Transcript Accumulation Index
TGFβ......................................................Transforming growth factor beta
TIP60…………………………………………………………………………………Tat interactive protein 60

TLR4………………………………………………………………………………Toll like receptor 4

TNFα………………………………………………………………………………Tumor necrosis factor alpha

tPA………………………………………………………………………………Tissue plasminogen activator

µM………………………………………………………………………………micro Molar

XTT………………………………………………………………………………sodium 3’-[1-[(phenylamino)-
carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate
Disclaimer

Parts of this dissertation have been published after successful acceptance in Scientific Reports and Journal of Alcoholism and Drug Dependence. The references are below:


CHAPTER 1. INTRODUCTION

1. Introduction

According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), 15.1 million adults in the United States suffer from alcohol use disorders (AUDs). The grave morbidities of AUDs range from an increased risk for certain cancers to death. One of the most severe yet under-studied mechanisms is the effect of alcohol on the human immune system. Several studies have revealed that alcohol abuse negatively affects the innate and adaptive immune systems, making them more vulnerable to infection (Szabo and Saha, 2015, Molina et al., 2010). Specifically, studies focused on the innate immune system have shown that, chronic alcohol abuse can cause skewed peripheral NK (natural killer) cell development and maturation (Zhang et al., 2017) along with severe intestinal permeabilization leading to cirrhosis (Keshavarzian et al., 1999b). Alcohol has also been shown to cause severe dysfunction in leukocyte recruitment during infections which can cause easily treatable infections to get severe (Zhang et al., 2002). Furthermore, alcohol abuse can deeply alter the functionality of one of the primary antigen presenting cells of the innate immune system, known as dendritic cells, by altering their stimulatory ability (Szabo et al., 2004), or by modulating cytokine and chemokine secretion at the bronchoalveolar region in case of cigarette smokers (Burnham et al., 2013), in hepatocytes and kupfer cells in case of chronic alcohol intoxication (Dong et al., 2016) or in case of dendritic cells from alcohol user (Nair et al., 2015).

Over recent years, alcohol exposure related studies have found molecular pathways like involvement in GABAergic and NMDA receptor pathways in the brain (Ron and Barak, 2016, Alfonso-Loeches and Guerri, 2011). Additionally, inflammation and
oxidative stress related pathways in the periphery (Louvet and Mathurin, 2015) have also shown to be profoundly associated with the effects of alcohol in the human brain and body. However, much still remains to be elucidated with regards to alcohol’s mechanisms of action in the human body, especially in the human immune system.

One of the emerging fields of study in the substance abuse research area is epigenetics. Epigenetics is the combined study of histones, histone post-translational modifications, DNA methylations and non-coding RNA based mechanisms that collaborate to alter gene expression or genetics (Huang et al., 2014). Epigenetic studies have paved the road to understanding several complex medical disorders and have introduced a new realm of epigenetic biomarkers and drug targets (Huang et al., 2014, Helin and Dhanak, 2013). The study of epigenetics has its own implications in alcohol research, and understanding the epigenetic mechanisms and pathways of alcohol has shown much potential and continues to remain a promising field of research (Jangra et al., 2016, Krishnan et al., 2014).

Alcohol has also been associated with the modulation of cannabinoid receptors in the human body (Nair et al., 2015, Pava and Woodward, 2012, Kleczkowska et al., 2016, Vasiljevik et al., 2013, Chanda et al., 2013, Ortega-Alvaro et al., 2015, Al Mansouri et al., 2014, Agudelo et al., 2013). Cannabinoid receptors (CB₁, CB₂) are endogenous receptors which were first studied in the context of endocannabinoids and CB₁ was found to be present primarily in the brain (Matsuda et al., 1993) and CB₂ primarily in the immune system (Munro et al., 1993, Felder and Glass, 1998). However, over the years, CB₁ has been found in peripheral tissues like cardiovascular and reproductive systems while CB₂ has been found in the central nervous system and neurons (Svíženská et al., 2008). The association of alcohol with the endocannabinoid system has been studied at length along
with cannabinoid receptors being studied as potential therapeutic drug targets for Alcohol Use Disorders (AUDs) (Onaivi, 2008, Pava and Woodward, 2012). Currently, with the existence of novel cannabinoid receptors like GPR55 (Ryberg et al., 2007), which are also being referred to as “type 3” cannabinoid receptors (Yang et al., 2016), newer studies targeted towards finding the therapeutic potential of such novel cannabinoid receptors are on the rise (Shore and Reggio, 2015b, Morales and Jagerovic, 2016). Alcohol has recently also been shown to modulate GPR55 in the brain (Serrano et al., 2012) and in the periphery (Agudelo et al., 2013). Therefore, the question behind the advantages of the cannabinoid receptors especially novel GPCRs like GPR55, and their usefulness as drug targets for AUDs remains to be answered.

1.1 Alcohol use disorder and its public health impact

According to the National Institutes on Alcohol Abuse and Alcoholism (NIAAA), AUD is a resulting effect of chronic problem drinking. Alcohol consumption can be classified as acute, chronic, binge drinking. Acute drinking refers to one episode of binge drinking causing acute effects of alcohol-like intoxication and chronic levels refer to more than one episode of moderate or binge drinking resulting in chronic effects of alcohol like dependence, chronic inflammation, and even cancer among other effects (Faden and Goldman, 2005). Car crashes due to driving under influence (DUI) can occur due to acute or chronic alcohol drinking. Another grave co-morbidity of alcohol consumption is that of fetal alcohol spectrum disorder which happens in children whose mothers were consuming alcohol during pregnancy (Riley et al., 2011). According to the 2015 National Survey on Drug Use and Health (NSDUH), 15.1 million adults ages 18 and older had an AUD. This includes 9.8 million men (8.4 percent of men in this age group) and 5.3 million women (4.2 percent of women in this age group) (NSDUH, 2015). In 2010, alcohol misuse cost the
United States $249.0 billion towards this preventable cause of morbidity and death (Sacks et al., 2015). According to the World Health Organization in 2014, alcohol consumption lead to more than 200 diseases and injury-related health conditions including liver cirrhosis and cancer (Organization and Unit, 2014). According to a study, more than 10 percent of the children in the United States live with a parent who has alcohol drinking problems (NIAAA, 2012). When it comes to treatments for AUDs, most treatments are based on therapy to control addiction behavior to alcohol (Yoon and Petrakis, 2018). Similarly drugs like naltrexone, acamprosate and disulfiram help in dealing with addiction (Yoon and Petrakis, 2018). However, more specific and efficient pharmacological treatment plans towards AUDs need to be studied. In conclusion, AUD continues to remain a public health concern and more active and effective research needs to continue to contribute towards establishing cures or treatments for AUDs.

1.2 Alcohol metabolism – an overview

Before going into details about molecular mechanisms of alcohol, this section will provide a brief outline of how alcohol is metabolized in the human body. After alcohol ingestion, almost all of it that is absorbed is metabolized in the liver (Lieber, 1997). Elimination of alcohol depends on several factors like gender (Cole-Harding and Wilson, 1987), age, race (Reed et al., 1976) or exercise patterns (Cederbaum, 2012) and diet (Ramchandani et al., 2001). In hepatic cells, alcohol can be metabolized by three different mechanisms. It can either be metabolized by alcohol dehydrogenase or ADH in the cytosol or it can undergo oxidation in the microsomes or it can be metabolized by catalase enzymes in peroxisomes (Caballería, 2003). All these mechanisms form acetaldehyde as one of the products which also happens to be the most toxic amongst all alcohol metabolites (Caballería, 2003). One of the main limitations of alcohol metabolism by ADH is the availability and hence capacity of ADH (Cederbaum, 2012). For example, availability
of ADH is more in the liver when alcohol is consumed after food intake than in fasting conditions (Cederbaum et al., 1977). Acetaldehyde is further metabolized to acetate in the mitochondria with the help of acetaldehyde dehydrogenase or ALDH which is ultimately released into the blood to be converted or oxidized further to simple molecules like carbon dioxide, water, and fatty acids (Caballería, 2003, Cederbaum, 2012). For both of these reactions by ADH and ALDH, NAD$^+$ is reduced to NADH + H$^+$ which when it occurs during binge alcohol exposure conditions leads to altered NAD$^+$/NADH ratio in the cells (Veech et al., 1972). This altered ratio especially in the mitochondria affects other reactions like mitochondrial oxidation as it affects the availability of NAD$^+$/NADH which leads to ROS generation (Veech et al., 1972). The highest amount of cytochrome P450 group enzymes is present in microsomes, amongst which, CYP2E1 has the highest affinity for metabolizing alcohol to acetaldehyde, this process eventually becomes responsible for producing the maximum amount of reactive oxygen species making it an important player in alcohol-related diseases with an oxidative stress component (Cederbaum, 2012).

1.3 Molecular mechanisms of action of alcohol in the human brain and periphery

Alcohol's mechanism of action is a topic that has attracted a lot of researchers and still continues to remain questionable. We have recently published a review paper (Parira et al., 2017d) that also discusses the same. Several researchers have incorporated different cellular and animal models to understand the mechanisms behind addiction behavior due to alcohol by studying brain cells and tissues in vitro or in vivo under acute and chronic alcohol conditions. When it comes to understanding the effect of alcohol in the brain, while acute alcohol exposure causes changes or temporary modifications for hours, chronic alcohol exposure causes an alteration in synapses and changes in overall circuitry (Most et al., 2014). Researchers have studied the effects of alcohol in the context of different neurotransmitter systems. An important target for alcohol studies is N-methyl-
D-aspartate (NMDA) receptors that are mostly associated with the glutamatergic pathways involved in learning and memory (Morris, 2013, Wang and Peng, 2016). For instance, there is some evidence that alcohol utilizes NMDA receptors for its effects through protein kinase C (Li et al., 2005). In vitro, acute alcohol treatment on hippocampus reduces NMDA activity correlating to levels that result in intoxication in humans and is directly proportionate to levels of intoxication (Lovinger et al., 1989). Dopamine and dopamine receptors have also been demonstrated to be affected by alcohol as rats that are bred to prefer alcohol produce more dopamine than wild-type rats in a study using an animal model of alcohol self-administration (Weiss et al., 1993). In a rat model of chronic intermittent ethanol exposure, ethanol was found to almost completely damage the signaling from D2/D4 and it also disrupted the cognitive function of the medial prefrontal cortex (Trantham-Davidson et al., 2014). Ras-specific guanine-nucleotide releasing factor 2 (RASGRF2) was found to control dopaminergic downregulation in the brain of RASGRF2 knockout mice after sub-chronic alcohol treatment (Easton et al., 2014). Finally, the Gamma-aminobutyric acid or GABA receptor has also been deeply associated towards the functionality of alcohol in brain (Maccioni and Colombo, 2009). GABA A receptors in dorsal raphe nucleus were shown to be responsible for alcohol-induced aggression with GABA A receptor agonist Muscimol further escalating this aggression in presence of alcohol but having no effect in absence of it (Takahashi et al., 2010). Interestingly a review paper discussed the role of G protein-coupled receptors or GPCRs like cannabinoid receptor 1 or CB1 and GABA B receptor in modulating or regulating alcohol-induced GABA release (Kelm et al., 2011).

On the other hand, several groups have also studied alcohol’s debilitating effect in the human periphery and peripheral organs like the liver and immune system as reviewed by Molina et al. (Molina et al., 2010). Alcoholic liver injury or liver cirrhosis involves
interaction between many pathways and cell types. Under the effect of alcohol, liver macrophages or Kupfer cells are sensitized and become vulnerable to endotoxin or bacterial lipopolysaccharide (LPS) which is considered as a hallmark of alcoholic liver disease (Mandrekar and Szabo, 2009). Toll-like receptor 4 (TLR4) on macrophages and other cell types recognizes LPS in the liver which causes downstream activation of several signaling pathways like the MAP kinases ultimately leading to activation of transcription factors like NFkB (Mandrekar and Szabo, 2009). These pathways lead to inflammation and generation of reactive oxygen species (Cederbaum et al., 2009). Leaky gut due to alcohol abuse has been shown to be a possible causative factor for alcohol-induced liver injury (Keshavarzian et al., 1999a). When it comes to the innate immune system, alcohol severely affects monocytes, macrophages, dendritic cells and natural killer cells amongst others (Molina et al., 2010). Several studies have shown that alcohol adversely affects different functional properties of monocytes by reducing their phagocytic ability and cell-cell adherence (Morland et al., 1988). Alcohol has also been shown to increase expression of certain receptors like CCR5 on macrophages which makes them more susceptible to HIV and SIV infections (Marcondes et al., 2008, Bagby et al., 2006, Bagby et al., 2003). Alcohol abuse also negatively affects all components of the innate immune system specifically the inflammatory response depends on whether the exposure was acute or chronic. Studies on human monocytes and rodent macrophages have shown that acute alcohol exposure causes a decrease in TLR responses which blunts the TNFa response, however, this effect wears off with repeated or long-term alcohol exposure and long-term alcohol exposure leads to loss of TLR4 tolerance (Bala et al., 2012) (Mandrekar et al., 2009a). In the context of DCs, alcohol has been shown to negatively affect exogenous antigen presentation by DCs by reducing the amount of peptide-MHCII complexes that are present on DCs, it also altered co-stimulatory molecules expression on the DC’s (Eken
et al., 2011). Chronic ethanol exposure was also found to damage migratory capacity of cutaneous DCs modulating skin immunity (Parlet and Schlueter, 2013). Another study which looked into the mechanism by which DC function of co-stimulation was affected by chronic alcohol exposure, DCs derived from mice post chronic alcohol feeding were found to express lower amounts of costimulatory molecules like CD40, CD80 or CD86 and produce less cytokines IL12-p40, TNFalpha and IFNalpha compared to mice that were water fed (Fan et al., 2011).

When it comes to the adaptive immune system, alcohol has been shown to adversely affect it causing immune dysregulation. The result of such immunodeficiency leads to increased vulnerability to bacterial pneumonia, tuberculosis, and other kinds of infectious diseases (T., 1998). Chronic alcohol exposure negatively affects almost all modules of the adaptive immune system (Pasala et al., 2015). Peripheral DC cells from alcoholics without liver disease patients were shown to have significantly reduced HLA-DR expression along with an increase in inflammatory cytokines secretion like IL-6, IL-12 and TNFα (Laso et al., 2007). Several studies performed in both humans and animal models of chronic alcohol drinking showed it reduced the quantity of T cells, alters the balance between the different subtypes, affects their activation and functioning and in the end also promotes apoptosis (Pasala et al., 2015).

1.4 Epigenetic mechanisms of alcohol action

Epigenetics, which encompasses three broad topics of DNA methylation, histone modifications, and micro RNA mediated mechanisms of gene regulation, has been in the spotlight of research in trying to understand the mechanism of action of different substances (Huang et al., 2014). Similarly, in the case of alcohol, many studies have looked at the epigenetic effects of alcohol in order to understand its mechanism of action.
Our recently published review paper discusses such epigenetic mechanisms in detail (Parira et al., 2017d). Especially in the case of histone modifications which was the focus of this dissertation. Previous studies on alcohol and histone modifications, originated with rat hepatocytes being treated with alcohol in a dose and time-dependent manner and reported that acetylation at the 9th lysine residue on histone H3 was upregulated after 24 hours of alcohol exposure (Park et al., 2003). Other studies have also shown differential effects of alcohol in the liver such as increased acetylation at H3K9, increase in phosphorylation at serine 10 and 28 among others (Park et al., 2005, Lee and Shukla, 2007). Apart from acetylation, phosphorylation at H3 ser 10 and 28 and phosphoacetylation at K9S10 have also been shown to be associated with expression of early response genes like c-fos, c-jun and MAP kinase phosphatase-1 in the liver of rat models of acute alcohol exposure (James et al., 2012). Studies of acute alcohol exposure in the brain showed an increase in histone H3 and H4 acetylation in rat amygdala (Pandey et al., 2008), while chronic intermittent alcohol exposure increased H3K9ac and H4K12ac in the frontal cortex region (Pascual et al., 2009). In a study using ethanol-conditioned rats, reduced alcohol consumption and preference was observed when the animals were injected with HDAC inhibitor Valproic acid (Al Ameri et al., 2014). Nonetheless, most of the studies described above focused on the brain or liver, leaving the body’s immune system especially specific and vulnerable cell populations like dendritic cells understudied. More on this topic will be discussed in Chapter 3 section 3.1, with emphasis on the studies carried out previously by us.

Apart from histone modifications, the role of alcohol towards modulating DNA methylation status had been studied prior. Acetaldehyde, a metabolite of alcohol had been shown to modulate DNA methylation by blocking DNA methyltransferases (Chen et al., 2011). In a model of alcohol dependency, post dependent rats showed increased drinking
associated with increased DNA methylation correspondingly decreasing neurotransmitter-related genes in the medial prefrontal cortex (Barbier et al., 2015). A study found binge-like alcohol to block DNA methylation in neural stem cells altering their functionality and adversely affecting differentiation by distressing critical genes like insulin-like growth factor 1 (Igf1), in cases of eye development, lens intrinsic membrane protein 2 (Lim 2); the epigenetic mark Smarca2 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2); and developmental disorder DiGeorge syndrome critical region gene 2 (Dgcr2)(Zhou et al., 2011). In a study of FASD, children born with FASD were found to have unique DNA methylation defects accompanied by changes in genes related to protocadherins, glutamatergic synapses, and hippo signaling (Laufer et al., 2015). Tissue plasminogen activator or tPA is one of the primary proteins in astrocytes responsible for neuronal plasticity and found to be increased by ethanol which was similar to an effect under DNMT inhibitor (Zhang et al., 2014b). Further, DNMT 3A but not DNMT 1 was found to be reduced in astrocytes after alcohol exposure (Zhang et al., 2014b). These studies further revealed tPA as a regulatory protein in the ethanol-DNA methylation axis.

Alcohol has also widely been shown to modulate the micro RNA landscape in humans. Several studies have shown the role of alcohol in modulating different micro RNAs as reviewed by Szabo and Bala (Szabo and Bala, 2013). Acute binge alcohol drinking was found to induce miR-27a expression in human monocytes which in turn modulated expression of ERK signaling that in turn affected monocyte activation and polarization (Saha et al., 2015). In another study miR-339-5p was found to prevent alcohol induced brain inflammation via NFκB signaling pathway in microglial cells isolated from alcohol exposed brain tissue (Zhang et al., 2014c). Some specific micro-RNAs are also emerging to be markers for certain liver diseases including alcoholic liver diseases like
micro RNA 122 (Zhang et al., 2010). Additionally, some micro RNA based mechanisms of alcohol have also been associated with the GABA receptor hence deepening the understanding of epigenetic effects of alcohol (Sathyan et al., 2007).

1.5 The endocannabinoid system

The endocannabinergic system consists of endocannabinoids (anandamide and 2-arachidonoylglycerol), cannabinoid receptors (traditional CB₁ and CB₂ and novel ones like GPR55), enzymatic degradation systems, (through the fatty acid amide hydrolase or FAAH enzyme for anandamide and the monoacylglycerol lipase enzyme for 2-arachidonoylglycerol) and cannabinoid transport reuptake system (Le Foll and Tyndale, 2015). CB₁ receptors which are primarily present in the brain (Devane et al., 1988), while CB₂ receptor have been shown to be present more in the periphery (Munro et al., 1993). However, many studies thereafter have reported CB₁ receptors to be present in periphery (Tam et al., 2010) and CB₂ receptors have been discovered in the brain (Onaivi et al., 2006), their levels however are lower in these locations. (Mechoulam and Parker, 2013).

In case of cannabinoid receptors as therapeutic targets, more focus has been put on CB₂ receptors since compounds modulating CB₂ eliminate the brain related effects (Dhopeshwarkar and Mackie, 2014). A recent review discusses how CB₂ receptor agonists have been shown to be immunosuppressive and have an immense future in therapeutic applications for autoimmune diseases and graft rejection (Eisenstein and Meissler, 2015). GPR55 which has been referred to as type 3 or novel cannabinoid receptor (Yang et al., 2016) has also been discussed to show their possible therapeutic potential (Shore and Reggio, 2015b). While GPR55 has been reported to couple to Gα13, Gα12, or Gαq proteins with activation of PLC, RhoA, ROCK, ERK, p38 mitogen-activated protein kinase, and Ca²⁺ release that can induce downstream transcription factors such as Nuclear factor of activated T-cells (NFAT), nuclear factor kappa-light-chain-enhancer
of activated B cells (NF-κB), CAMP Responsive Element Binding Protein (CREB), and Activating Transcription Factor 2 (ATF2) (Shore and Reggio, 2015a), CB₁ and CB₂ show biased signaling in the sense that relative activation of Goi and Goo is dependent on the agonist leading to either activation or inhibition adenylyl cyclase with further activation of MAP kinases (Ibsen et al., 2017).

1.6 Alcohol and the endocannabinoid system

Alcohol’s association with cannabinoid receptors and the cannabinergic pathway has been and is still being studied quite extensively. Several studies in rodent models have shown cannabinoid agonists and antagonists to have an effect on alcohol self-administration. In a study in mice model with a two-bottle choice paradigm, CB₂ agonist β-caryophyllene showed a dose-dependent decrease in alcohol preference and drinking (Al Mansouri et al., 2014). Chronic ethanol exposure has been shown to down-regulate CB₁ receptor function (Basavarajappa and Hungund, 2002). In another study, when adolescent rats were exposed to alcohol were compared to controls, CB₁ receptor and nuclear PPARα were found to be lower in spleens; From alcohol-fed animals however, CB₂ was higher showing that exposure to alcohol can bring about significant differences in cannabinergic receptor expression (Pavon et al., 2016). In yet another study in mice with ATG5 (autophagy related gene) knockout model, researchers showed that CB₂ receptor activation in liver macrophages protects them from steatosis in an autophagy-dependent pathway (Denaes et al., 2016). In a recent interesting study, the researchers found that acute alcohol exposure in humans increased CB₁ in the cerebrum and this effect was reversed in chronic alcohol exposure that is again maintained for a time period of at least a month (Ceccarini et al., 2014). Recently interaction between CB₂ and social environment was shown to modulate chronic alcohol consumption (Pradier et al., 2015). In this study, a mice model was used where CB₂ knockout mice were compared to wildtype with
different social environment groups of single or grouped housing and forced or two bottle choice paradigms for alcohol drinking (Pradier et al., 2015). Interestingly, the study showed CB₂ knockout mice were drinking more when housed in groups and given an intermittent forced drinking paradigm (Pradier et al., 2015). Further studies especially the ones previously published by us will be described later in Chapter 4, section 4.1. We have also published a review paper discussing the alcohol cannabinergic pathway association and the studies showing epigenetic basis of these interactions (Parira et al., 2017d).
CHAPTER 2: HYPOTHESIS AND RESEARCH QUESTIONS

2.1 Objective of the Study

The goal of this study was to understand the epigenetic effects of chronic alcohol treatments in human MDDCs and find out the epigenetic mechanisms behind alcohol's modulation of cannabinoid receptors.

2.2 Hypothesis:

The hypothesis of the study is that chronic alcohol treatment modulates human MDDC function through epigenetic mechanisms.

2.3 Research questions:

The study was divided into three questions:

a) How does chronic alcohol treatment modulate epigenetic modifications in human MDDCs?

b) Do these epigenetic changes under chronic alcohol treatment modulate human MDDC function?

c) What are the mechanisms that underlie the functional effects exerted by chronic alcohol exposure of human MDDCs?

The following sections will discuss in details each question individually by giving a short introduction to each, detailing the methods and results and then discussing the results. The dissertation will finally conclude with a common conclusion and future directions section.
CHAPTER 3: RESEARCH QUESTION 1: How does chronic alcohol treatment modulate epigenetic modifications in human MDDCs?

1.1 Introduction

In our laboratory, we have previously published about the role of histone deacetylases (HDACs) in human monocyte-derived dendritic cells (MDDCs) and neuronal cells under acute alcohol exposure and MDDCs from alcohol users. In neuronal cells, SK-N-MC, acute treatment with alcohol showed increased expression of class I HDACs and a corresponding increase in ROS production (Agudelo et al., 2011, Agudelo et al., 2012). We have recently shown that in in vitro studies acute alcohol exposure is able to modulate class I HDACs, specifically upregulating gene expression of HDAC 1,2,3 and 8 (Agudelo et al., 2016). Further, in MDDCs derived from alcohol users, there was also a significant increase in gene expression of all class I HDACs and protein expression of HDAC 2 and 8 (Agudelo et al., 2016). Additionally, there was an increase in total HDAC activity in alcohol users compared to non-users while gene expression array and in silico analysis for the class I HDACs also showed modulation of oxidative stress-related genes (Agudelo et al., 2016).

Additionally, preliminary data for HDAC activity and expression of HDACs either did not show significant differences or showed lower levels compared to control for MDDCs treated chronically with alcohol, and since a decrease in HDAC activity or lack of activity might result in an increased in acetylation, for this project we decided to focus on looking deeper into histone post-translational modifications focused at acetylations in MDDCs due to chronic alcohol exposure. Histone post-translational modifications being more intrinsic in the epigenetic pathways and closer to modulating gene expression were selected in
order to elucidate alcohol-induced epigenetic and cannabinergic association. All data under this section have been published recently (Parira et al., 2017b).

3.2 Methods

3.2.1 MDDC Isolation – Human MDDCs were isolated from commercially available buffy coat from community blood bank (One Blood, FL). In brief, blood was diluted with PBS and white blood cell layer was separated using density gradient separation. White blood cell layer was pipetted out, washed with PBS and residual red blood cells were lysed using Ammonium-Chloride-Potassium (ACK) lysing buffer (Quality biologicals). Resulting peripheral blood mononuclear cells (PBMCs) were plated with RPMI media (Gibco) and after 2 hours floating cells were discarded while adherent monocytes were washed and cultured for 5 to 7 days with cytokines 100 U/mL IL-4 and 100 U/mL GM-CSF (R & D systems) to differentiate into MDDCs.

3.2.2 Chronic alcohol treatment and Measurement of alcohol concentration – Initially, to optimize alcohol concentrations and to confirm daily dose of alcohol, a separate experiment was performed to check daily alcohol concentrations. Post differentiation, MDDCs were treated with alcohol (Ethanol or EtOH) 0.2% (200 g/dL or ~40mM) for 5 days. Similar alcohol concentration was used in previous studies (Liang et al., 2014b). Alcohol-treated cells were kept in a separate incubator humidified with alcohol and the treatment was replenished in full every day. As alcohol is known to evaporate up to 90% after 24 Hours in neuroblastoma x glioma cells (Rodriguez et al., 1992). Our method of chronic alcohol treatment has been validated previously in monocytes where replenishing alcohol every day and keeping cells in alcohol-humidified incubator maintained concentration at 25mM±4.5 over a 7 day period (Mandrekar et al., 2009b). Further this technique of treatment has been described by the same group previously (Szabo and
Mandrekar, 2008). Alcohol concentration within the culture media was checked starting at 48 hours then every 24 hours after that using a kit (Abnova) that measures color of product chromate converted from dichromate, a reaction that utilizes ethanol/alcohol, pre replenishment and 2 hours post replenishment with alcohol.

### 3.2.3 Histone extraction and analysis of histone quantity and modifications ELISA

- total histones were extracted using a total histone extraction kit (Epigentek) and total H3 and H4 were quantified using H3 and H4 quantification kit using an ELISA based method of quantification of histones. H3 and H4 modifications were quantified using H3 modification multiplex array (EpiQuik Histone H3 Modification Multiplex Assay Kit, Epigentek) and H4 modification multiplex array (EpiQuik Histone H4 Modification Multiplex Assay Kit, Epigentek) kits. In brief, equal amounts of protein were added for different samples in every experiment; however, overall protein concentration range from 100 to 200 ng. H3 modifications were calculated according to manufacturer’s instructions which also accounts for protein amounts and the final values for each modification were presented as a percentage over untreated control. H4K12ac was also measured in histone extracts using H4K12ac quantification kit (Epigentek).

### 3.2.4 H4K12ac western blot and Mass spectrometry analysis -

Western blotting was carried out with 2 µg of total histone extracts from control cells and alcohol treated cells. They were electrophoresed on a 4–20% polyacrylamide gel (BIORAD) and labelled with primary antibody rabbit anti-human H4K12ac (EMD Millipore) (1:2000) and Horseradish peroxidase (HRP) labelled secondary antibody, anti-rabbit IgG (1:2000) (EMD MILLIPORE). Loading was controlled by normalizing H4k12ac blot intensities to histone H4 by labelling H4 with anti-human H4 (1:10,000) (Thermo Scientific™) and HRP labelled secondary anti-mouse IgG (1:10,000). Films were analyzed using Image J software.
Quantification of H4 acetylation levels in controls and alcohol treated samples was validated using MALDI-FT-ICR-MS in collaboration with mass spectrometry facility at the Department of Chemistry and Biochemistry. In brief, all the sample preparation was performed in our lab including histone extraction, histone quantification, and separation by SDS-PAGE followed by coomasie blue staining of gel. H4 bands were isolated from the gel at the Mass Spectrometry facility for paid service of the H4 purification and analysis. The procedure is available in details in published manuscript (Parira et al., 2017b).

3.2.5 NU9056 treatment and single cell imaging flow cytometry - Post differentiation, MDDCs were pre-treated with 50nM NU9056 for 1-2 hours with replenishing treatment every 48 Hours along with media change (concentration selected based on viability studies, data available in manuscript (Parira et al., 2017b)). Cells that receive alcohol treatment were treated with 0.2% EtOH for 5 days. Post chronic alcohol treatment, cells were harvested and stained with fixable viability dye eFLUOR450 (eBioscience) to gate on viable cells only. Further, they were intra-nuclearly stained with a primary anti-H4K12ac antibody (EMD Millipore) and secondary anti-rabbit FITC labeled antibody (EMD Millipore) to visualize the localization and expression of H4K12ac labeled cells. Post-staining, cells were acquired by Amnis FlowSight, 10,000 live single cell images were acquired and data were analyzed through Ideas analysis software.

3.2.6 Statistical Analysis - Data are expressed as mean ± standard error of mean (Xu et al.). Experiments were performed at least three times or otherwise indicated in the figure legends. Statistical analysis like student’s t-test and/or 1-way or 2-way ANOVA were done using either Microsoft Excel or GraphPad Prism software. For more than one treatment groups, post-hoc analysis like Tukey’s multiple comparisons test was also carried out to
test for significance. Values of the same have been expressed as F (DFn, DFd). P values were considered significant when they were ≤ 0.05.

3.3 Results

3.3.1 Chronic alcohol treatment regime maintains 0.2% alcohol concentration in human MDDCs. Post differentiation, MDDCs were harvested and treated with 0.2% EtOH and kept in an incubator humidified with same concentration of ethanol. EtOH concentration was assayed in the cell culture media on days 2, 3, 4 and 5 before and 2 hours after replenishment. On measurement of alcohol concentration before replenishment, we saw almost 90% evaporation every 24 hours (data not shown). Similar evaporation rates have also been observed previously (Rodriguez et al., 1992, Hurley et al., 1987). As shown in Figure 1, after replenishing the EtOH levels come back up close to 0.2%. Hence daily replenishing of EtOH and incubating cells in EtOH incubator help maintain desired EtOH concentration in cells.

3.3.2 Chronic alcohol treatment significantly increases histone H3 and H4 quantity in human MDDCs. Post chronic alcohol treatment, total histones were extracted from EtOH treated and untreated or control MDDCs. Isolated histones were analyzed for quantity of total histone H3 and H4 using ELISA based technique. As shown in figure 2, total H3 was significantly upregulated after 0.1% EtOH (111.6 ± 4.858, p = 0.033) and 0.2% EtOH (138.1 ± 15.18, p = 0.027) while total H4 was significantly upregulated only at 0.2% EtOH (214.5 ± 33.02, p = 0.0017) when compared to untreated control. 0.1% EtOH treated MDDCs only increased H3 significantly but not H4 compared to untreated MDDCs. For this reason, for the following modification array studies, H3 modification array was carried out for both 0.1% and 0.2% EtOH treated MDDCs while H4 modification array was carried out for only 0.2% EtOH treated MDDCs (Parira et al., 2017a).
3.3.3 Chronic alcohol treatment significantly decreases most histone H3 and H4 post-translational modifications but significantly increases acetylation at 12th lysine on H4 (H4K12ac). Post chronic alcohol treatment, total histones were extracted from EtOH treated and untreated or control MDDCs. Isolated histones were analyzed for quantity of 21 H3 post-translational modifications and 10 H4 post-translational modifications using ELISA based modification arrays. Results of H3 modifications as shown in figure 3, show a significant downregulation compared to untreated MDDCs. H3K4me1 (82.78% ± 2.95, p = 0.004), H3K4me3 (87.25% ± 3.67, p = 0.025), H3K9me3 (85.97% ± 5.17, p = 0.053), H3K27me2 (83.54% ± 6.30, p = 0.059), H3K36me3 (78.63% ± 7.17, p = 0.041) and H3K18ac (83.62% ± 3.80, p = 0.013) were expressed at lower levels in comparison to untreated MDDCs. However, treatment with 0.1% EtOH did not significantly modulate H3 modification sites. While most H4 modifications show lower levels compared to untreated MDDCs such as H4K5ac (15.44% ± 28.31, p = 0.011), H4K8ac (33.69% ± 12.89, p = 0.0002), H4K20m2 (53.63% ± 10.55, p = 0.0009), H4K20m3 (59.63% ± 10.55, p = 0.019), H4R3m2 (33.45% ± 10.27, p = 0.0003) and H4ser1P (49.36% ± 19.86, p = 0.025) in comparison to untreated MDDCs. Surprisingly, the only H4 modification that show significantly higher levels compared to untreated control was H4K12ac (401.64% ± 123.40, p = 0.031). Based on these data, the following experiments were carried out to validate the alcohol-induced increase of H4K12ac in treated MDDCs compared to untreated control (Parira et al., 2017a).

3.3.4 Chronic alcohol treatment induction of H4K12ac was further validated by western blot and mass spectrometry analysis (samples for mass spectrometry was acquired in our lab followed by mass spectrometry analysis carried out by FIU mass spectrometry facility). Post chronic alcohol treatment, total histones were extracted from EtOH treated and untreated or control MDDCs. Western blot experiments show MDDCs
treated chronically with 0.2% EtOH exhibited a significantly higher expression of H4K12ac (367.3 % ± 132.9, p = 0.05) in comparison to control or untreated MDDCs (100% ± 15.05). H4K12ac was normalized to total H4 as carried out in previous publications (Gaikwad et al., 2010, Esse et al., 2013). As shown in Fig. 4, the control and the 0.2% EtOH-treated MDDCs have K16 acetylated as indicated by the m/z 530.3045 and peptide 5-18 signal with higher relative abundances on the acetylated peptide in the treated MDDCs compared to an acetylated peptide in the control MDDCs (1.12 +/− 0.16 times) (panel d). Furthermore, a peptide signal was detected containing acetylation at K12 and K16 at m/z 927.5365 peptide 10-18 (panel d) for the control and treated MDDCs, respectively, with higher relative abundances in the treated compared to the control MDDCs (1.73 +/− 0.12 times) (panel d). A peptide signal was detected at m/z 1396.8026 peptide 14-18 (panel d) for the control and treated MDDCs corresponding to the acetylation of K5, K12, and K16; in this case, a higher relative abundance was also observed for the treated MDDCs compared to the control MDDCs (1.39 +/− 0.09 times) (panel d). These observations suggest higher levels of acetylation in MDDCs treated chronically with 0.2% EtOH compared to the untreated MDDCs. This study further confirms the upregulation of H4K12ac under chronic alcohol treatment (Parira et al., 2017a).

3.3.5 Chronic treatment with histone acetyltransferase inhibitor NU9056 (50nM) significantly blocks alcohol-induced upregulation in H4K12ac. In order to further understand the role of H4K12ac, blocking of alcohol-induced H4K12ac was needed. For this purpose, according to literature, amongst other histone acetyltransferases, TIP60 was found to be most responsible for acetylation of H4K12 (Altaf et al., 2010, Wee et al., 2014). Further, a novel TIP60 inhibitor, NU9056 was being investigated for its effects and role in human neuronal cells (Tan et al., 2016a). Based on the literature, NU9056 was selected and viability assay like trypan blue and XTT assay was carried out to select 50nM as an
optimum concentration for treatment in human MDDCs (data available in publication Parira et al, 2017c). Post differentiation, MDDCs were pre-treated with 50nM NU9056 for 2 hours and then cells receiving alcohol treatment were treated with 0.2% EtOH for five days. Post-treatment, either cells were immune-labeled with fluorescent H4K12ac through intra-nuclear labeling or histone extracts were prepared for H4K12ac quantification using an ELISA based kit. The results have been shown in figure 5. As measured by ELISA shown in panel a, chronic exposure of MDDCs with 50 nM NU9056 (69.35 ± 15.96) reduces H4K12ac lower than basal levels compared to control (100 ± 22.65) while 0.2% EtOH treatment (138.2 ± 18.09, p = 0.05) significantly increases H4K12ac compared to control. Also, when the cells were co-treated with 0.2% EtOH and NU9056 (50 nM) (56.27 ± 31, p = 0.04), there was a significant reduction on H4K12ac compared to 0.2% EtOH-treated cells, confirming the ability of NU9056 to inhibit the effects of EtOH on H4K12ac. To further validate the ability of NU9056 to inhibit the effect of EtOH on H4K12ac, intra-nuclear staining to detect H4K12ac followed by single-cell imaging flow cytometry was carried out. Figure 5, panel b shows a representative colored histogram overlay of the intensity of channel 2 (H4K12ac-FITC). For isotype control (yellow), secondary antibody only (orange), control (red), NU9056 50 nM (pink), 0.2% EtOH (blue) and combination of 0.2% EtOH and NU9056 50 nM (green) treated MDDCs. The histogram overlay represents the shift in intensity of H4K12ac positive cells showing a decrease or leftward shift for NU9056 (50 nM), increase or rightward shift for 0.2% EtOH compared to control and a decrease again for the combination of 0.2% EtOH and NU9056 (50 nM) in comparison to 0.2% EtOH-treated MDDCs. Panel d shows NU9056 (50 nM) (62.78% ± 3.53, p = 0.004) significantly reduced the percentage of H4K12ac positive cells compared to control (74.93% ± 1.73) while 0.2% EtOH increased the percentage of H4K12ac positive cells (82.95% ± 0.84, p = 0.0008) compared to control. When the cells
were treated with a combination of 0.2% EtOH and NU9056 (50 nM), there was a significant reduction on H4K12ac positive cells (68.21% ± 2.57, p = 0.0005) compared to cells treated with 0.2% EtOH alone. Panel c shows the mean fluorescent intensity (MFI) of H4K12ac-FITC positive cells. MDDCs treated with NU9056 50 nM (154451 ± 25304) shows reduced MFI compared to untreated MDDCs (172842 ± 28155). MDDCs chronically treated with 0.2% EtOH (203686 ± 41517) show increased MFI compared to control while MDDCs treated with 0.2% EtOH and NU9056 50 nM (133163 ± 23359, p = 0.0002) show significantly reduced MFI compared to 0.2% EtOH treated MDDCs. The results from panel d confirm that NU9056 50 nM in combination with 0.2% EtOH reduce alcohol-induced H4K12ac expression in MDDCs. Representative single cell images are shown in panel e. To confirm the nuclear co-localization of H4K12ac, treated or untreated MDDCs were labeled with H4K12ac using intra-nuclear staining protocol and followed by staining with 4’,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI). Images were acquired, and panel f shows representative single cell images with the last channel showing an overlay of bright field, FITC and DAPI, confirming the nuclear co-localization of H4K12ac (Parira et al., 2017a).

3.4 Discussion

Epigenetics is a newly emerging field that is slowly gaining importance in understanding mechanisms of how various compounds or substances and even conditions and diseases function (Mann, 2014, Qureshi and Mehler, 2013, Stylianou, 2013, Cao et al., 2014). In this study, we attempted to understand the epigenetics of chronic alcohol treatments in modulating functionality of human MDDCs. We first measured total histone quantity (histone H3 and H4) to understand if chronic alcohol treatments had any effect on overall histone levels. Our results showed chronic treatment with 0.2% EtOH significantly increased total histone H3 and H4 quantity in MDDCs.
compared to untreated MDDCs (Figure 2). These results are consistent with other findings since histone quantity has been previously measured and found to be modulated due to deletion of High Mobility Group Box 1 protein (HMGB1) in mouse embryo fibroblasts and due to differentiation of mouse embryonic stem cells (Celona et al., 2011, Karnavas et al., 2014b). As discussed in our recently published paper (Parira et al., 2017a), Celona et al., have shown that histone content is reduced in the cells which makes the chromatin more accessible and open to transcription (Celona et al., 2011). Another group has shown that histone quantity is increased in embryonic stem cells that are differentiating and which is also a symbol of pluripotency (Karnavas et al., 2014a). In our study, we observed an increase in histone content which may correlate with the MDDC differentiation stage and maturation effects induced by chronic exposure to EtOH (Parira et al., 2017a).

Having seen increased H3 and H4 quantity, we assessed H3 and H4 post-translational modification status in MDDCs post chronic alcohol treatment. Out of 21 different H3 modifications that were quantified, most H3 modifications that showed significant differences were downregulated (Figure 3). A total of 10 different H4 modifications were quantified and similarly most H4 modifications showed downregulation except acetylation at H4K12 or H4K12ac. We further confirmed our finding using western blot and mass spectrometry (collaboration with the MS facility at FIU). As discussed in our publication (Parira et al., 2017a) previous literature also shows an increase in H4K12ac in adolescent rats due to chronic intermittent alcohol exposure which is further correlated with increased oxidative stress (Pascual et al., 2009). In another report, in a transgenic model of Alzheimer’s disease, H4K12ac was found to be increased and correlated with an increase in inflammatory cytokines like MIP-2 and TNFα (Plagg et al., 2015). Additionally, in a study with pancreatic cancer, pancreatic duct adenocarcinoma tumor samples from patients showed H4K12 and H3K18 acetylations to have a high correlation with tumor
stage indicating them as prognostic markers (Juliano et al., 2016). It was recently observed that high H4K12ac expressing tumor samples from patients with oral squamous cell carcinoma correlated with gender, alcohol consumption and cervical lymph node metastasis (Campos-Fernandez et al., 2018). Based on this literature and from results which show an increase in H4K12ac after chronic alcohol exposure, it can be concluded that upregulated H4K12ac could serve as an important epigenetic marker during chronic alcohol abuse (Parira et al., 2017a).

In order to further understand the function of H4K12ac in MDDCs under alcohol exposure, we assayed a newly identified compound NU9056 which was found to inhibit histone acetyltransferase TIP60 (Coffey et al., 2012). TIP60 has been previously shown to be responsible for histone H4 acetylation specifically modulate H4K12ac amongst other acetylation sites (Sanchez-Molina et al., 2014). Therefore, after assaying for the viability and toxicity of various concentrations of NU9056 (Parira et al., 2017a), we selected 50nM and measured H4K12ac in MDDCs treated chronically with 0.2% EtOH, 50nM NU9056 or both and demonstrated that NU9056 could inhibit EtOH-induced H4K12ac and bring it back to basal or control levels.

To summarize, chronic alcohol treatments in human MDDCs, increase total histone H3 and H4 quantity and while alcohol downregulates most H3 and H4 modifications, it upregulates H4K12ac. TIP60 inhibitor NU9056 is able to inhibit EtOH-induced H4K12ac and bring it back down to basal levels. The significance of this study lies in the fact that H4K12ac may be developed and further studied to be an epigenetic marker or therapeutic target for AUD’s and NU9056 may be explored as a therapeutic drug target.
Figure 1: Chronic alcohol treatment regime maintains 0.2% alcohol concentration in human MDDCs.

Post differentiation, during chronic alcohol treatment, alcohol concentration was measured on days 2, 3, 4 and 5, post replenishment with 0.2% Alcohol. The alcohol concentration is represented as mean % ± SEM.
Figure 2: Chronic alcohol treatment significantly increases histone H3 and H4 quantity in human MDDCs.

After 5–7 days of differentiation, MDDCs were treated with 0.1%, and 0.2% alcohol for 5 days. Panel a: Histone H3 and H4 quantity was analyzed by ELISA-based EpiQuik quantification kits independent of its modified state. The values are displayed as percent of control ± SEM from at least five independent experiments. Graph indicates H3 at EtOH 0.1% (111.6 ± 4.858, p = 0.033) and 0.2% (138.1 ± 15.18, p = 0.027), H4 at EtOH 0.2% (214.5 ± 33.02, p = 0.0017). 1-way ANOVA was performed to show no significant difference between treatments, F (4, 46) = 1.842, P=0.1370. Post-hoc analysis with Tukey’s multiple comparisons test showed no significant differences. Statistical differences were calculated using student’s t-test when each treatment was individually compared to untreated control and 1-way ANOVA followed by Tukey’s multiple comparisons test was carried out to test for significance between treatments. Significant differences are indicated with * for p≤0.05.
FIGURE 3

a. 

H3 modifications (% of Control)

- Control
- H3K4me1
- H3K4me3
- H3K9me3
- H3K27me2
- H3K36me3
- H3K18ac

- EtOH 0.1%
- EtOH 0.2%

b. 

H4 modifications (% of Control)

- Control
- H4K5ac
- H4K8ac
- H4K12ac
- H4K20me2
- H4K20me3
- H4K20me1
- H4K16ac
- H4K16me2
- H4K16me1

- * indicates significant difference
Figure 3: Chronic alcohol treatment significantly decreases most histone H3 and H4 post-translational modifications but significantly increases acetylation at 12th lysine on H4 (H4K12ac).

Panel (a) Histone extracts from MDDCs chronically treated with EtOH 0.1 and 0.2% were analyzed for 21 H3 post-translational modification states. Results show levels of significantly altered histone modifications for 0.1 and 0.2% EtOH, presented as percentage over untreated control ± SEM from two (EtOH 0.1%) and three (EtOH 0.2%) independent experiments. Chronic treatment with 0.2% EtOH in MDDCs showed significant downregulation at the sites of H3K4me1 (82.78% ± 2.95, p = 0.004), H3K4me3 (87.25% ± 3.67, p = 0.025), H3K9me3 (85.97% ± 5.17, p = 0.053), H3K27me2 (83.54% ± 6.30, p = 0.059), H3K36me3 (78.63% ± 7.17, p = 0.041) and H3K18ac (83.62% ± 3.80, p = 0.013) in comparison to untreated control. 2-way ANOVA was performed to test for significance between treatments and showed significant column factor F (1, 21) = 9.18, P=0.0064. Post-hoc analysis with Tukey's multiple comparisons test did not show any significant differences. Panel (c): Histone extracts from MDDCs chronically treated with EtOH 0.2% were analyzed for 10 H4 post-translational modification states. Results show levels of significantly altered histone modifications for EtOH 0.2% presented as percentage over untreated control ± SEM from 7 (EtOH 0.2%) independent experiments. Chronic treatment of the EtOH 0.2% in MDDCs showed significant downregulation at H4K5ac (15.44% ± 28.31, p = 0.011), H4K8ac (33.69% ± 12.89, p = 0.0002), H4K20m2 (53.63% ± 10.55, p = 0.0009), H4K20m3 (59.63% ± 10.55, p = 0.019), H4R3m2 (33.45 ± 10.27, p = 0.0003) and H4ser1P (49.36 ± 19.86, p = 0.025) and upregulation at H4K12ac (401.64% ± 123.40, p = 0.031), in comparison to untreated control. 2-way ANOVA was performed to test for significance between treatments and showed significant column factor F (7, 42) = 7.388, P<0.0001. Post-hoc analysis with Tukey's multiple comparisons test was carried out and it showed significant difference between control and H4K12ac (P=0.001). Statistical differences were calculated using student’s t-test when each treatment was individually compared to untreated control and 2-way ANOVA followed by Tukey's multiple comparisons test was carried out to test for significance between treatments. Significant differences are indicated with * for p≤0.05.
FIGURE 4

a.

b.

Optical Density of H4K12ac (normalized to H4 control)

Treated/Control

CHRONIC

5-18 GLGKGGAKR
10-18 GLGKGGAKR
14-18 GLGAKR
Figure 4: Chronic alcohol induction of H4K12ac was further validated by western blot and mass spectrometry analysis (post histone sample preparation in lab, Mass spectrometry experiment and analysis was carried out at the MS facility at FIU).

Western blot was carried out for H4K12ac and H4 in histone extracts from untreated control or 0.2% EtOH treated MDDCs. Panel a- shows representative blot highlighting the protein of interest where first 3 lanes have histone extracts from three biological replicates of untreated MDDCs and next 3 lanes contain histone extracts from three biological replicates of MDDCs chronically treated with 0.2% EtOH. The H4K12ac band appears at 11 kDa while the H4 band appears at 14 kDa. Panel b - optical density of H4K12ac normalized to H4 when analyzed by ImageJ and represented as % control. MDDCs chronically treated with 0.2% EtOH (367.3 %± 132.9, p = 0.05) showed a higher expression of H4K12ac in comparison to untreated MDDCs (100 %± 15.05). Western blot was carried out with histone extracts for control and 0.2% EtOH treated MDDCs from at least 13 different buffy coats. Statistical differences were calculated using t-test when compared to untreated control. Typical representative MALDI-FT-ICR MS spectra of the 10–18 GLGKGGAKR peptide has been shown in Panel c, for control and 0.2% EtOH treated MDDC samples (theoretical isotopic pattern is shown for all cases). There is higher abundance of the acetylated peptides in the chronic 0.2% EtOH-treated MDDCs relative to the control MDDCs (Panel d). The experiment was carried out in 36 µg of total histone extracts pooled from untreated and 0.2% EtOH treated MDDCs.
Figure 5: Chronic treatment with histone acetyltransferase inhibitor NU9056 (50nM) significantly blocks alcohol-induced upregulation in H4K12ac.

Post differentiation, MDDCs were treated with NU9056 50 nM, EtOH 0.2% or both for 5 days. Panel a shows H4K12ac quantity from histone extracts as percent (%) of control ± SEM from at least three independent experiments. Statistical differences were calculated using student’s t-test compared to untreated control (100 ± 22.65) and indicated for H4K12ac at NU 50 nM (69.35 ± 15.96), EtOH 0.2% (138.2 ± 18.09, p = 0.05), and EtOH 0.2% + NU 50 nM (56.27 ± 31, p = 0.04). Interactions between groups were tested by 1-way ANOVA which gave a F(3,28)=2.494, p = 0.08. Intra-nuclear staining for H4K12ac and single cell imaging flow cytometry were also performed and it was carried out at least three times in triplicates. 10,000 events were acquired from live population (eFLUOR450 dye) per sample. Controls with secondary (2°) antibody (Ab) only and isotype staining were also analyzed. Panel b shows a representative overlay of histogram of intensity of channel 2 (FITC) for each treatment shown in legend on the histogram. Panel d shows the percentage of FITC positive (H4K12ac expressing cells). 2° Ab control showed (0.02% ± 0.009) H4K12ac positive population. Isotype control showed (0.34% ± 0.001) positive for H4K12ac. NU 9056 (50 nM) (62.7% ± 3.5, p = 0.004) reduced H4K12ac positive cells compared to control (74.93% ± 1.73) while 0.2% EtOH increased H4K12ac positive cells (82.9% ± 0.84, p = 0.0008) compared to control. 0.2% EtOH and NU9056 (50 nM) reduced H4K12ac positive cells (68.21% ± 2.57, p = 0.0005) compared to cells treated with 0.2% EtOH alone. 2-way ANOVA gave a significant value of F(3,30)=14.42, p <0.0001. Panel c shows MFI of H4K12ac population from each treatment after background subtraction. 0.2% EtOH + NU9056 50 nM (133163 ± 23359, p = 0.0002) show significantly reduced MFI compared to 0.2% EtOH (203686 ± 41517) treated MDDCs only. 2-way ANOVA showed significant row factor (F(10,30)=36.77, p<0.0001) and significant column factor (F(3,30)=8.72, p=0.0003). Panel e shows representative single cell images from experiments where live cells were acquired based on staining with fixable viability dye eFLUOR 450. Panel f shows representative single cell images confirming nuclear staining with H4K12ac antibody based on DAPI co localization with H4K12ac.
CHAPTER 4: Research Question 2: Do these epigenetic changes under chronic alcohol treatment modulate human MDDC function?

4.1 Introduction

Dendritic cells, the sentinels of the human immune system, professional antigen presenting cells are crucial in initiating and determining immune responses (Boltjes and van Wijk, 2014). The primary functions of dendritic cells lie in antigen uptake and presentation with the help of cell surface markers and receptors, production of cytokines and chemokines and finally launching an immune response to foreign antigens (O’Keeffe et al., 2015). As previously discussed, several studies have shown alcohol exposure to modulate their functional properties but how epigenetic changes due to alcohol exposure modulate DC functionality is not completely understood. Previous researchers have studied certain epigenetic mechanisms of DC function, for example, lysine methyl transferases (KMT) especially KMT1c is an important component towards monocyte differentiating to DC’s (Wierda et al., 2015). Another study showed that histone modifications of H3K4me3 and H3K27me3 may be an important player towards DC differentiation and function in a tumor like or inflammatory environment (Mei et al., 2014). To further understand the functional relevance of alcohol induced H4K12ac towards MDDC functionality, this aim will look at cell surface markers and receptor expression on MDDCs under alcohol exposure and how the cytokine or chemokine secretions by MDDCs get modulated by the same.

As previously elaborated in the introduction cannabinoid receptors have been indicated to be involved with mechanisms of alcohol action. However, when it comes to understanding the epigenetic basis of this interaction, not much is known in that aspect. A few studies have explored to gain a deeper understanding of this association from
epigenetics point of view. One such study on fetal alcohol spectrum disorder (FASD) showed that, when CB₁ receptor was knocked out in neonatal mice, they were protected from alcohol’s debilitating effects on DNA methyltransferases DNMT1, DNMT 3A and overall DNA methylation (Nagre et al., 2015). Another study in a mouse model of FASD showed ethanol exposure caused upregulation of H4K8 acetylation at the CB₁ receptor exon further causing neurobehavioral abnormalities in mice (Subbanna et al., 2014).

Previous reports from our lab have shown acute alcohol exposure was able to increase expression of cannabinoid receptors CB₂ and GPR55 in primary human MDDCs (Agudelo et al., 2013). Additionally, in MDDCs derived from alcohol users, CB₂ and GPR55 were found to be increased in expression compared to MDDCs derived from non-users (Agudelo et al., 2013). To further understand the epigenetic basis of this interaction between alcohol and cannabinoid receptors in MDDCs, the newly established role of H4K12ac under chronic alcohol exposure was studied for its ability to modulate cannabinoid receptor expression. These studies were first carried out at acute levels using ChIP-PCR and reducing acetylation at H4K12 through NU9056 and siRNA, and results showed that after acute alcohol levels, H4K12ac was associated with an increase in GPR55 expression in primary human MDDCs treated acutely with alcohol (Castillo-Chabeco et al., 2018). However, whether alcohol’s modulation of H4K12ac was associated with regulation of cannabinoid receptor expression even after chronic alcohol treatments was yet to be studied.

4.2 Methods

4.2.1 Cell surface marker study for chronic alcohol treatment – To understand if chronic treatment with alcohol is causing dendritic cells to differentiate and mature further, MDDCs were immune-phenotyped for monocyte surface marker CD14 (Ziegler-Heitbrock
and Ulevitch, 1993) and dendritic cell surface marker CD11c (Singh-Jasuja et al., 2013). They were labeled with an anti-CD14-APC cy7 antibody (eBioscience) and anti-CD11c-APC antibody (eBioscience) followed by acquisition with FlowSight single cell imaging flow cytometer (Amnis, EMD Millipore). Data were analyzed using Ideas image analysis software.

### 4.2.2 Cytokine array and MCP2 ELISA
The expression of 48 inflammatory cytokines was analyzed according to manufacturer instructions with the RayBiotech inflammation arrays (RayBiotech, Norcross, GA, USA) as previously described by us (Agudelo et al., 2013, Parira et al., 2017c, Parira et al., 2017a). Briefly, supernatants from cells after 5-day post-treatment with NU9056, 0.2% EtOH and 0.2% EtOH + NU9056 and an untreated control were assayed for the experiment. MCP-2 ELISA was carried out using MCP-2 ELISA kit (RayBiotech) as per manufacturer’s instructions (Parira et al., 2017a).

### 4.2.3 Gene quantification of GPR55.
Total RNA was isolated from treated or untreated MDDCs using commercially available DNA/RNA/Protein isolation TriplePrep kit (GE healthcare). Following isolation, RNA was reverse transcribed to cDNA (Agilent Technologies) and cDNA was used to amplify GPR55 and 18srRNA using primers GPR55 (Hs00271662_s1, Thermofisher Scientific) and 18srRNA (#4333760F, Thermofisher Scientific). Relative mRNA species expression was quantitated and the mean fold change in expression of the target gene was calculated using the comparative CT method and expressed as Transcript Accumulation Index or TAI. 18s rRNA was used as endogenous control.

### 4.2.4 Protein quantification of GPR55: western blotting and single cell imaging flow cytometry
Total protein was isolated from treated or untreated MDDCs using commercially available RNA/Protein isolation kit (GE healthcare). GPR55 protein
expression was measured by western blotting with 30ug of protein isolate, using anti-GPR55 antibody (Bios) normalized to GAPDH (Sigma-Aldrich). GPR55 was also quantified by single-cell imaging flow cytometry. 1 × 10⁵ cells were stained with AF-647-labeled GPR55 antibody (Bios). Rabbit isotype control antibody (Thermo Fisher) was also used. Intra-cellular staining for H4K12ac was carried out using Fix/Perm buffer kit (BD Biosciences) following the manufacturer’s protocol. Cells were first stained with fixable viability dye eFLUOR®450 (eBioscience) to gate on live cell population during image acquisition. Image acquisition by Amnis® FlowSight® Imaging Flow Cytometer and analysis by IDEAS® image software was carried out as previously described by us (Figueroa et al., 2016, Parira et al., 2017c) (Parira et al., 2017a). For each experiment, from all events collected, AF647 positive cells were gated from single cells.

4.2.5 Treatment with CID and NU9056 and GPR55 protein quantification. Treatment with NU9056 was carried out according to a previously elucidated method and concentration. Optimum concentration for the GPR55 antagonist, CID16020046 (CID) (Tocris) was selected by XTT viability and Lactate dehydrogenase toxicity assay (Supplementary Figure S1). MDDCs were pre-treated with 5 uM CID followed by chronic treatment with 0.2% EtOH for MDDCs receiving both treatments. Post chronic treatment, GPR55 was quantified using single cell imaging flow cytometry. Data were analyzed as previously elucidated.

4.2.6 Statistical analysis. Data are expressed as mean ± SEM. Experiments were performed at least three times or otherwise indicated in the figure legends. Statistical analysis like student’s t-test and 1-way and/or 2-way ANOVA were done using either Microsoft Excel or GraphPad Prism software. For more than one treatment groups, post-hoc analysis like Tukey’s multiple comparisons test was also carried out. Values of the
same have been expressed as $F$ (DFn, DFd). $P$ values were considered significant when they were $\leq 0.05$.

4.3 Results

4.3.1 Chronic alcohol treatment does not affect the population of dendritic cells in late stage of differentiation.

During chronic alcohol treatment, expression levels of monocytic marker CD14 and dendritic cell marker CD11c was assessed by single-cell imaging flow cytometry. They were measured at Day 0 before starting alcohol treatment, followed by measurement at Day 3 and Day 5 post-treatment. Concurrent with our previous publication (Figueroa et al., 2016), as seen in figure 6 panel a, purely monocytic cell population or CD14 positive population form the smallest percentage of the total cell population. Even during chronic treatment, at Day 3 and Day 5 CD14 positive population remain under 10% of the total population with no significant differences between alcohol-treated and untreated MDDCs. As shown in panel B, dendritic cells in early stages of differentiation characterized by CD11c and CD14 double positive cell population are significantly high in 0.2 % EtOH treated MDDCs at both Day 3 (26.9% ± 0.7, $p=0.03$) and Day 5 (21.2% ± 1.6, $p=0.02$) compared to untreated MDDCs on Day 3 (14.8% ± 3.7) and Day 5 (12.9 % ± 1.9) respectively. As shown in panel C, the majority of cell population (57.4%) fall under dendritic cells that are in the late stage of differentiation as characterized by CD11c positive and CD14 negative and almost 80% of cell population is CD11c positive hence dendritic cells in either early or late stages of differentiation. However, there is no significant difference between 0.2% EtOH treated and untreated MDDCs at Day 3 or 5 of treatment.
4.3.2 Inhibition of H4k12ac with NU9056, modulates cytokines and chemokines induced by alcohol treatments in human MDDCs. To understand the role of chronic alcohol treatment and H4K12ac in inflammation in MDDCs, 48 inflammatory cytokines and chemokines were studied by using an inflammation array. Cell culture supernatants from untreated MDDCs or MDDCs treated EtOH, NU9056 or both were analyzed for secreted inflammatory cytokines and chemokines. As shown in figure 7, panel a, a graphical representation of optical density values show treatment with 0.2% EtOH significantly upregulates ICAM-1 (4.71 ± 0.42, p = 0.0007) and IL-10 (5.008 ± 0.89, p = 0.027). While inhibition of H4K12ac by NU9056 (50 nM) significantly upregulates ICAM-1 (7.37 ± 1.16, p = 0.002), IL-6 (4.55 ± 1.21, p = 0.015), IL-10 (4.13 ± 1.03, p = 0.046), IL-15 (4.33 ± 1.84, p = 0.062), IL-16 (2.62 ± 1.02, p = 0.059) and IP-10 (4.15 ± 0.69, p = 0.002) when compared to untreated control. In addition, when the cells were treated with a combination of 0.2% EtOH and NU9056, there was a significant upregulation of ICAM-1 (10.43 ± 2.42, p = 0.011), IL-10 (4.35 ± 0.55, p = 0.023), IL-15 (6.68 ± 1.01, p = 0.0009), IL-16 (1.94 ± 0.35, p = 0.0004) and IP-10 (3.95 ± 0.35, p = 0.0002) when compared to untreated control. Statistical analysis between MDDCs co-treated with 0.2% EtOH + NU9056 versus MDDCs treated with only 0.2% EtOH revealed a significant upregulation of IL-15 (p = 0.036), RANTES (p = 0.014), TGFβ-1 (p = 0.0008) and TNFα (p = 0.028) as shown in figure 7 panel c, and significant downregulation of MCP-2 (p = 0.016) as shown in figure 7, panel d. Representative blots for each treatment have been shown in panel b. To further validate the modulation of MCP-2 by H4K12ac, MCP-2 levels were measured by ELISA (panel e) in cell culture supernatants. The combination of 0.2% EtOH and NU9056 50 nM (321.64 pg/mL ± 13.50, p = 0.03) significantly reduced MCP-2 levels compared to 0.2% EtOH treatment alone (401.75 pg/mL ± 28.22). (Parira et al., 2017a)
4.3.3 Chronic alcohol treatments increase gene and protein expression of GPR55.

In order to understand the effect of chronic alcohol treatment on GPR55 gene and protein expression, post-treatment, cells were either stained for single cell imaging flow cytometry or total RNA and protein was isolated to perform gene expression studies through qPCR and protein expression studies through western blotting respectively. Panel a shows transcript accumulation index of GPR55 measured by qPCR analysis where 0.2% EtOH treated MDDCs (1.64±0.24, p=0.02) show significantly increased GPR55 gene transcription compared to untreated MDDCs. To confirm GPR55 upregulation in MDDCs chronically treated with 0.2% EtOH, western blot was performed using total protein extracts and GPR55 protein levels from treated samples and controls were normalized to GAPDH. Panel b shows the optical density of GPR55 normalized to GAPDH where 0.2% EtOH (1.7±0.1, p=0.0006) shows significantly higher GPR55 protein expression compared to untreated MDDCs (0.9±0.06). Panel c shows a representative blot picture. As shown in panel d, 0.2% EtOH treated MDDCs have a significantly higher percentage of cells (59.7%±5.5, p=0.0005) expressing GPR55 compared to untreated MDDCs (30.8%±4). Panel e shows a representative overlay histogram of the intensity of GPR55. Representative single cell images are shown in Panel f.

4.3.4 GPR55 antagonist CID16020046 reduces chronic alcohol-induced protein expression of GPR55. Since chronic alcohol treatment was able to increase the GPR55 gene and protein expression, we wanted to further understand the effect of blocking GPR55 expression. In order to block GPR55 expression, we selected GPR55 antagonist CID16020046 (CID) which was found to inhibit LPI induced NFAT, NFκB, calcium release and GPR55 internalization in HEK293 cells (Kargl et al., 2013). We carried out viability and toxicity studies (Supplementary Figure S1) to select an optimum concentration of 5uM of CID. Post-treatment of MDDCs with 0.2% EtOH, or 5uM CID or combination of both,
immunostaining with anti-GPR55-AF647 was carried out and GPR55 expression levels were measured using single cell imaging flow cytometry. As shown in Figure 9 panel a, MDDCs treated with 0.2% EtOH + 5uM CID (12.4±3.8%, p=0.0001) significantly reduces percentage of GPR55 positive cells compared to 0.2% EtOH treated MDDCs only (59.7%±5.5). Thus, this data showed the ability of combination treatment of EtOH and CID to reduce EtOH induced GPR55. Panel b shows a representative overlay histogram of GPR55 intensity. Panel c shows representative single cell images.

4.3.5 *H4K12ac antagonist NU9056 decreases chronic alcohol-induced protein expression of GPR55*. To further understand if acetylation at H4K12 is associated with GPR55 expression, we carried out GPR55 quantification studies after MDDCs were treated with HAT inhibitor NU9056 and EtOH or combination of both. Post-treatment of MDDCs with 0.2% EtOH, or 50nM NU9056 or combination of both, immunostaining with anti-GPR55-AF647 was carried out and GPR55 expression levels were measured using single cell imaging flow cytometry. As shown in Figure 10 panel a, treatments with 0.2% EtOH + 50nM NU9056 (14.4±8.3%, p=0.0001) significantly reduces the percentage of GPR55 positive cells compared to 0.2% EtOH-treated MDDCs only (59.7%±5.5). Thus, these data showed the ability of combination treatment of EtOH and NU9056 to block EtOH induced GPR55. Panel b shows a representative overlay histogram of GPR55 intensity. Panel c shows representative single cell images.

4.4 Discussion

In order to understand how chronic alcohol treatments modulate MDDC functionality, we first assessed the purity of the dendritic cells that were differentiated from monocytes and then treated chronically with alcohol. Other studies have shown alcohol treatments to modulate differentiation and maturation of immune cells (Zhang et al., 2015,
Saha et al., 2015). We have previously shown CD11c a dendritic cell marker and CD14 a monocytic cell marker (Figueroa et al., 2016) as effective cell surface markers to understand the purity of dendritic cells in case of processes that differentiate dendritic cells from monocytic cells (Wolkow et al., 2018). CD11c on antigen presenting cells has been shown to interact with Th17 cells and help in expression of inflammatory chemokines (Paterka et al., 2016). CD11c along with CD11b has also been shown to play a role in DC tolerance (Wang et al., 2016). Amongst several functions of CD14 like serving as coreceptor for TLR4 and enabling cellular response to low dose of LPS (Zanoni and Granucci, 2013), recently it has been elucidated to initiate inflammosome dependent phagocytosis after incurring inflammatory lipids (Zanoni et al., 2017). Our results showed (Figure 6), that about 80% of cells are CD11c+ hence dendritic cells in the total cell population. About 60% were in the late stages of differentiation (CD11c+ CD14-) and there was no significant difference between those cell populations. About 20% were in the early stages of differentiation (CD11c+ CD14+) which showed a significant difference between treatments. Hence, even though there may be some variations due to studies which have dendritic cells in the early stages of differentiation or monocytic cells included in them, it may be outweighed by the majority of dendritic cells in the late stages of differentiation. Overall purely monocytic cells or CD14+ forms only about 10% of the total population of cells and CD11c- CD14- cells form about 10% of the total population of cells used for the experiment hence the effects due to the same may be negligible. Previous studies have reported chronic alcohol exposure for 3 months in rhesus macaques reduced number of myeloid dendritic cells (HLA-DR+CD11c+CD123-) in both PBMCs and bone marrow cells (Siggins et al., 2009). In yet another study, BALB/c mice that were fed ethanol for 11 days showed increased CD11c on dendritic cells and decreased IL-6, IL-12, IL-17A, and IFN-gamma and increased IL-13 cytokine production in response to ovalbumin stimulation.
An interesting clinical study showed men living with HIV and concomitant alcohol use showed increased presence of soluble CD14 in blood plasma (Monnig et al., 2016). Another study reported that chronic alcohol use reduced CD14+CD16- subset of peripheral monocytes and increased CD14dimcd16+ subset, overall increasing IL-6 and TNF production (Donnadieu-Rigole et al., 2016). These studies further show alcohol associated modulation in monocytic/dendritic cell surface markers and modulation of function of the same.

To understand if H4K12ac serves an inflammatory role, we measured 48 inflammatory cytokines and chemokines in MDDCs untreated or chronically treated with 0.2% EtOH, NU9056 or 0.2% EtOH + NU9056. NU9056 in combination with 0.2% EtOH treatment enhanced the effects of EtOH on cytokine production by upregulating the secretion of IL-15, RANTES, TGFβ-1 and TNFα compared to EtOH 0.2% while blocking the secretion of MCP-2 compared to 0.2% EtOH treatment, suggesting a partial inflammatory role of H4K12ac (Parira et al., 2017a). Histone acetylations are typically associated with increased gene and protein expression thereby positively regulating genes (Krebs and Peterson, 2000). A study showed, chronic binge alcohol increases gene expression of pro-inflammatory CCL2, CCL8, CX3CL1, SELE, HP, and TNFRS10A and expression of pro-fibrotic genes in the extra cellular matrix like TIMP-1, MMP 2, and MMP 9 mRNA in skeletal muscle tissue of SIV infected macaques (Dodd et al., 2014).

Alcohol has been shown to modulate cannabinoid receptors (Agudelo et al., 2013, Al Mansourri et al., 2014, Zheng et al., 2015); however, the mechanism behind this association is still being investigated. Some studies have explored epigenetic mechanisms behind this association (Subbanna et al., 2014, Nagre et al., 2015); however, many detailed studies are needed to understand the alcohol-cannabinergic pathway in-depth.
We recently published that chronic alcohol treatment was able to upregulate acetylation at H4K12 (Parira et al., 2017a) and acute alcohol treatment was able to upregulate H4K12ac at GPR55 gene region (Castillo-Chabeco et al., 2018). To further validate this and to understand the role of chronic alcohol treatment in GPR55 expression, we measured the GPR55 gene and protein expression through real-time qPCR, western blotting and single cell imaging flow cytometry. The results confirmed chronic alcohol treatment was able to upregulate gene and protein expression of GPR55 as previously demonstrated by us in MDDCs treated acutely with alcohol and MDDCs from self-reported alcohol users. Most commonly, increased histone acetylation corresponds to increase in gene transcription and further increased translation resulting in increased protein expression (Krebs and Peterson, 2000). Since increased acetylation of H4K12 is accompanied by increased GPR55 gene and protein expression it is possible that chronic alcohol treatment induced H4K12ac may be at least partially regulating expression of cannabinoid receptor GPR55. Previous reports have also shown increased intragenic histone acetylation to result in increased gene expression (Johansson et al., 2015). There may also be other factors that are contributing towards its upregulated expression like increased acetylation at promoter regions and other epigenetic factors like micro-RNAs. We further wanted to confirm the nature of this gene regulation. We tested the ability of GPR55 antagonist CID to be able to block chronic alcohol-induced GPR55 expression and found that even though CID by itself does not affect GPR55 protein expression (corroborated by previously published reports where CID prevented GPR55 internalization in HEK293 cells (Kargl, 2013 #433)) and keeps it at the same levels as untreated MDDCs, in combination with EtOH it decreases GPR55 expression compared to EtOH treated MDDCs (Figure 9). This decreasing effect may be explained by drug-drug interactions between CID and EtOH. Pharmacodynamic interactions between natural and synthetic
cannabinoids and drugs of abuse like alcohol has been shown to modulate overall interaction and functionality as reviewed recently (Arellano et al., 2017). Similar effects of decrease in GPR55 expression due to alcohol exposure in combination with GPR55 antagonist CBD was demonstrated in mice where CBD reduced alcohol induced hypothermia and handling issued convulsion (Viudez-Martinez et al., 2018). However, in order to understand how CID by itself does not reduce GPR55 expression but in combination with alcohol is able to reduce it needs further investigation.

Once we confirmed that CID revered/ reduced alcohol effects on GPR55, we wanted to study if alcohol-induction of GPR55 was due to acetylation or correlated to alcohol-induced H4K12ac; therefore we used previously studied and published TIP60/HAT inhibitor which was found to inhibit alcohol-induced H4K12ac (Parira et al., 2017a). We measured GPR55 protein levels using NU9056 alone or in combination with EtOH and found that in combination with EtOH it reduces GPR55 protein levels compared to EtOH treated MDDCs. This further confirmed the association between alcohol-induced H4K12ac and GPR55 expression. It may be noteworthy to mention here that even though NU9056 alone did not have a significant downregulation on GPR55 compared to untreated MDDCs, when compared to CID, it showed a lower level of GPR55 expression implying that blocking TIP60 causes a stronger blocking effect on GPR55 expression inhibiting alcohol-effects on GPR55. This further sheds some light on to the mechanism lying underneath this associative pathway. In conclusion, chronic alcohol treatment was able to upregulate H4K12ac with a concomitant increase in GPR55 gene and protein expression. Additionally, GPR55 antagonist CID was able to decrease alcohol-induced GPR55 and TIP60/HAT inhibitor NU9056 was able to reduce alcohol-induced GPR55. However further experiments are needed to understand this pathway in-depth. Some of the future directions are to carry out siRNA-mediated knockdown of TIP60 during chronic alcohol
treatment conditions, which may give more concrete answers to our questions. Analyzing H4K12ac levels at GPR55 promoter region using chromatin immunoprecipitation (ChIP) will also help us further understand its relationship with GPR55 gene regulation. When it comes to understanding the epigenetic status of GPR55 gene and promoter, not much is known in terms of histone post translational modifications but a recent report showed GPR55 promoter to have heavy DNA methylation regions in colon cancer cell lines with lower degree of methylation in the gene body and 5' UTR (Hasenoehrl et al., 2018). We were recently able to show upregulated H4K12ac at the gene region of GPR55 in MDDCs after acute alcohol exposure (Castillo-Chabeco et al., 2018), however we need to carry out these studies in MDDCs after chronic alcohol exposure to understand these epigenetic effects in depth.
Figure 6: Chronic alcohol treatment does not affect population of dendritic cells in late stages of differentiation.

Post differentiation of MDDCs, single cell imaging flow cytometry analysis for cell surface markers CD11c and CD14 was carried out at three time points. Day 0 (before starting chronic treatment, Day 3 and Day 5 of chronic alcohol treatment). 1 x 10^6 cells were stained with anti-CD11c (APC)-red (CH11) and anti-CD14 (APC-cy7)-pink(CH12) followed by staining with DAPI. Out of 10,000 images acquired, DAPI negative or live cell population was gated. From live cell population, CD14+, CD11c+CD14- and CD11c+CD14+ cells were gated utilizing scatter plots. Percent positive cells were graphed in panels a, b and c. Panel a shows CD14 positive or monocytic cell populations at day 0, 3 and 5. no significant differences were found. Panel b shows CD11c+CD14+ or early dendritic cells or dendritic cells in early stages of differentiation. Early dendritic cells characterized by CD11c and CD14 double positive cell population are significantly high in 0.2 % EtOH treated MDDCs at both Day 3 (26.9% ± 0.7, p=0.03) and Day 5 (21.2% ± 1.6, p=0.02) compared to untreated MDDCs on Day 3 (14.8% ± 3.7) and Day 5 (12.9 % ± 1.9) respectively. Panel c shows CD11c+CD14- or late dendritic cells or dendritic cells in late stages of differentiation. No significant differences were found. Panel d shows a representative histogram of intensity of DAPI and gating of alive cell population. Panel e shows representative single cell images. Panel f shows a representative scatter plot of CD11c or CH11 and CD14 or CH12 intensity with gating of four different subtypes of cell population indicated as CH11+CH12- (57.4%), CH11+CH12+ (24.9%), CH11-CH12+ (9.4%) and CH11-CH12- or double negative (8.3%). The experiments were performed from at least 3 different buffy coats. Significance was calculated by students T-test and indicated by * for p≤0.05.
1. IL-15, 2. MCP-2, 3. RANTES, 4. TGF-β1, 5. TNF-α
Figure 7: Inhibition of H4k12ac with NU9056, modulates cytokines and chemokines production by alcohol treatments in human MDDCs.

After 5-7 days of differentiation, MDDCs were treated with NU9056 50nM, 0.2% EtOH or both for 5 days. Supernatants collected were analyzed for 48 inflammatory cytokines and chemokines. Array blots were analyzed using ImageJ software. Panel a shows graphical representation of optical density (OD) values post background correction (subtracting of mean negative from OD) ± SEM. Represented data is from 3 blots for untreated control, 2 blots each for treatment with 0.2% EtOH alone, treatment with NU9056 alone, and treatment with 0.2% EtOH plus NU9056. Cytokines chosen to be presented in the graph were selected on the basis of fold change (2 folds or more) compared to control. Panel b shows representative blots with boxes highlight cytokines shown in panel c. Each cytokine is detected in duplicates within each blot. Panel c shows optical density values for cytokines IL-15, RANTES, TGF-β1 and TNF-α. Panel d shows optical density values for MCP2. Statistical analysis between MDDCs co-treated with 0.2% EtOH + NU9056 versus MDDCs treated with only 0.2% EtOH to reveal a significant upregulation of IL-15 (p = 0.036), RANTES (p = 0.014), TGFβ-1 (p = 0.0008) and TNFα (p = 0.028) as shown in panel c, and significant downregulation of MCP-2 (p = 0.016) as shown in panel d. Statistical differences were calculated using student’s t-test when individually compared to untreated control and indicated with a * (compared to control) and # (between treatments) for significant p value. Data is represented as Mean ± SEM. Two-way ANOVA was carried out to test for interaction giving a significant value (F(27, 120)=3.521, p<0.0001). In panel e, MCP-2 ELISA was carried out from cell culture medium at least three times in duplicates. Values detected in pg/mL are as follows, untreated control (322.4 ± 25.04), NU9056 50 nM (317.5 ± 14.3), 0.2% EtOH (401.7 ± 28.2) and 0.2% EtOH + NU9056 50 nM (321.6 ± 13.5, p = 0.03). The experiment was carried out at least thrice in duplicates. Two-way ANOVA was carried out to test for interaction which gave a significant column factor of F(3,21)=3.542, p=0.03. Data is represented as Mean ± SEM. Significant differences are indicated with * for p≤0.05.
MDDCs were chronically treated with 0.2% EtOH. Post 5 days, either total RNA and protein was isolated from the cells for qPCR and immunoblotting analysis respectively or single cell imaging flow cytometry was carried out. Panel a shows transcript accumulation index (Wang et al.) of GPR55 measured by qPCR analysis. 0.2% EtOH treated MDDCs (1.64±0.24, p=0.02) show significantly increased GPR55 gene transcription compared to untreated MDDCs. Panel b shows optical density of GPR55 normalized to GAPDH. 0.2% EtOH (1.7±0.1, p=0.0006) shows significantly higher GPR55 protein expression compared to untreated MDDCs (0.9±0.06). Panel c shows representative immunoblot. 1x10^6 MDDCs were stained with fixable viability dye eFLUOR450 and anti-GPR55-AF647 using intracellular cell staining protocol and single cell imaging flow cytometry was carried out. 10,000 live cell images were acquired using FlowSight and data was analyzed using Ideas Application where AF647 positive cells were gated from total single cells and represented as % gated cells in panel d. 0.2% EtOH treated MDDCs have a significantly higher percentage of cells (59.7%±5.5, p=0.0005) expressing GPR55 compared to untreated MDDCs (30.8%±4). Panel e shows representative overlay histogram of intensity of AF647 for all treatments. Panel f shows representative single cell images. All experiments were performed at least thrice. Data is represented as Mean ± SEM. Statistical significance was calculated using Students T-test with EtOH 0.2% compared to control, and p value considered significant at p≤0.05 and annotated as *. 
FIGURE 9

a. % Positive Population (GPR55)

b. Frequency of GPR55 intensity

<table>
<thead>
<tr>
<th>Bright Field</th>
<th>Side Scatter</th>
<th>DAPI</th>
<th>GPR55 / AF647</th>
<th>Overlay</th>
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<tr>
<td>Control</td>
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<tr>
<td>ETOH 0.2%</td>
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<tr>
<td>5uM CID</td>
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<td>EtOH 0.2% + 5uM CID</td>
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Figure 9: GPR55 antagonist, CID16020046, decreases chronic alcohol-induced protein expression of GPR55.

Post chronic treatment of MDDCs with 0.2% EtOH, or 5uM CID or combination of both, immunostaining with anti-GPR55-AF647 was carried out and GPR55 expression levels were measured using single cell imaging flow cytometry. As shown in panel a, MDDCs treated with 0.2% EtOH + 5uM CID (12.4±3.8%, p=0.0001) significantly reduces percentage of GPR55 positive cells compared to 0.2% EtOH treated MDDCs only (59.7%±5.5). 1-way ANOVA showed significant difference between treatments (F(3,40)=19.69, p<0.0001). Panel b shows representative overlay histogram of intensity of GPR55 or AF647 for all treatments. Panel c shows representative single cell images. The experiment was carried out at least thrice in duplicates. Data is represented as Mean ± SEM. Statistical differences were calculated using student’s t-test when each treated samples were individually compared to untreated control and 1-way ANOVA followed by Tukey’s multiple comparisons test to test for significance between treatments, significant differences are indicated with * for p≤0.05.
Figure 10: H4K12ac antagonist, NU9056, decreases chronic alcohol-induced protein expression of GPR55.

Post-treatment of MDDCs with 0.2% EtOH, or 50nM NU9056 or combination of both, immunostaining with anti-GPR55-AF647 was carried out and GPR55 expression levels were measured using single cell imaging flow cytometry. As shown in panel a, MDDCs treated with 0.2% EtOH + 50nM NU9056 (14.4±8.3%, p=0.0001) significantly reduces percentage of GPR55 positive cells compared to 0.2% EtOH treated MDDCs only (59.7%±5.5). 1-way ANOVA showed a significant difference between treatments (F(3,34)=12.78, p<0.0001). Panel b shows a representative overlay histogram of GPR55 intensity. Panel c shows representative single cell images. The experiment was carried out at least thrice. Data is represented as Mean ± SEM. Statistical differences were calculated using student’s t-test when each treated samples were individually compared to untreated control and 1-way ANOVA followed by Tukey’s multiple comparisons test to test for significance between treatments, significant differences are indicated with * for p≤0.05.
Supplementary Figure S1: XTT and LDH assay point towards CID5uM as optimum concentration for chronic treatments: Panel a: XTT assay show, 15uM CID (72±4.3%, p=0.0002) causes significantly reduced viability in MDCs compared to control (100±4.8%). Panel b: LDH assay show no significant differences between untreated and treated MDDCs. The values shown on the graph for LDH activity (mU/mL) are as follows: Positive control (1020±2.9), Control (30.4±1), 1uM CID (35.4±0.41), 2.5uM CID (38.5±0.2), 5uM CID (30.5±0.9), 10uM CID (32.3±0.8) and 15uM CID (22.5±0.1). All experiments were performed at least twice in duplicates. Statistical significance was calculated using Students T test and p value considered significant at p≤0.05 and annotated as *.
CHAPTER 5: Research Question 3: What are the mechanisms that underlie the functional effects of chronic alcohol exposure of human MDCDs?

5.1 Introduction

Amongst several adverse effects of alcohol abuse, inflammation, and oxidative stress have gained importance over time so much so that alcoholism has been referred to as a systemic proinflammatory condition (Gonzalez-Reimers et al., 2014). While acute alcohol inhibits the inflammatory response, chronic alcohol abuse enhances inflammatory responses (Szabo and Saha, 2015). Apart from enhancing expression of proinflammatory responses, alcohol has also been discussed to suppress anti-inflammatory cytokines (Szabo and Saha, 2015). Apart from inflammation, oxidative stress or production of reactive oxygen species (ROS) is often linked to inflammation and even towards exacerbating inflammation thereby contributing towards pathophysiology of several debilitating diseases (Lugrin et al., 2014). Alcohol abuse has also been shown to cause oxidative stress and ROS production through its metabolism or through inflammation which has been studied as one of the causes of alcoholic liver disease (Li et al., 2015).

Amongst different therapeutic drug targets for AUD’s, cannabinoids have been investigated for the same potential and have been discussed to have opposing neuro-immunomodulatory effects compared to alcohol (Nair et al., 2015). While CB2 receptor agonists have been shown to reduce pain and inflammation related adversities in chronic alcohol or high-fat diet-induced pancreatitis (Zhang et al., 2014a), the GPR55 antagonist CID has been shown to protect against intestinal inflammation (Stancic et al., 2015) and reduce chemo-resistance in pancreatic cancer cells (Singh et al., 2016). The GPR55 antagonist Cannabidiol (CBD) was also recently shown to reduce ethanol consumption, motivation and relapse in mice (Viudez-Martinez et al., 2018).
Alcohol users have shown upregulated inflammatory cytokines and chemokines compared to non-users or controls (Nair et al., 2015). Further, we have also previously shown that acute alcohol exposure upregulates ROS production in human neuronal cells (Agudelo et al., 2011). In this current chapter, we wanted to further study the mechanisms that underlie the functional effects of chronic alcohol exposure of human MDDCs. To keep this aim in mind, we studied MCP-2 levels further by single cell imaging flow cytometry. We further studied oxidative stress or ROS production in human MDDCs treated with NU9056. To focus further in the GPR55 cell signaling, we also studied cyclic AMP levels in MDDCs. In all assays, treatments of NU9056 were incorporated to understand the role of H4K12ac and CID was incorporated to understand the role of GPR55.

5.2 Methods

5.2.1 MCP2 single cell imaging flow cytometry 1 × 10^6 cells were stained with primary MCP2-FITC labeled antibody (Novus) following manufacturers protocol for the fixation/permeabilization kit (BD Biosciences). Cells were first stained with fixable viability dye eFLUOR®450 (eBioscience) to gate on live cell population during image acquisition. Image acquisition by Amnis® FlowSight® Imaging Flow Cytometer and analysis by IDEAS® image software was carried out as previously described by us (Figueroa et al., 2016, Parira et al., 2017c) (Parira et al., 2017a). For each experiment, from all events collected, FITC positive cells were gated from single cells.

5.2.2 Single cell imaging flow cytometry study of ROS Post chronic treatments (0.2% EtOH, NU9056 and CID for 5 days), MDDCs were harvested and aliquoted into 1 million cells per mL of medium for single cell imaging flow cytometry. MDDCs receiving NU9056 or CID were pre-treated with it for two hours followed by addition of 10 uM 2’,7’ – dichlorofluorescein diacetate (DCF-DA) (Sigma, St. Louis, MO). For positive control,
hydrogen peroxide (50µM H\textsubscript{2}O\textsubscript{2}) was added, and the untreated control cells received just media. ROS was measured through single-cell imaging flow cytometry, where post alcohol or H\textsubscript{2}O\textsubscript{2} treatment, viability dye DAPI was added to the cells, and 10,000 live single cell images per sample were acquired using Amnis FlowSight. Data were analyzed using Ideas software. DCF-DA positive cells were gated on total alive single cell images.

5.2.3 MHC Class II marker HLA-DR expression study through single-cell imaging flow cytometry Post differentiation, HLA-DR quantification using single cell imaging flow cytometry, was carried out at three different time points. On Day 0, before beginning chronic treatment, 1 x 10\textsuperscript{6} MDDCs were stained with an anti-HLA-DR-FITC labeled antibody (eBioscience) and DAPI and 10,000 events were acquired using Amnis FlowSight. Data were analyzed using Ideas software. HLA-DR - FITC positive cells were gated on total alive (DAPI negative) single cell images. Similarly, single cell imaging flow cytometry was carried out for MDDCs treated or untreated with EtOH on Day 3 and Day 5 of chronic treatment. Additionally, on Day 5, MDDCs chronically treated with NU9056 or CID and their combination with EtOH were also studied for HLA-DR expression.

5.2.4 cyclic AMP assay Cyclic AMP (cAMP) was measured using cAMP assay kit from R&D systems following the manufacturer’s protocol. In brief, post chronic treatment, cells were harvested divided into three groups for each treatment where one group received treatment with 100uM Forskolin (Tocris) for 15 minutes to induce cAMP, another group received 10mM Albuterol (Tocris) treatment for 15 minutes, while the other group did not receive any treatment. Post-treatment, cells were lysed, and cell lysates were assayed for levels of cAMP according to manufacturer’s instructions.

5.2.5 Statistical analysis Data are expressed as mean ± SEM. Experiments were performed at least three times or otherwise indicated in the figure legends. Statistical
analysis like student’s t-test and/or 1-way or 2-way ANOVA were done using either Microsoft Excel or GraphPad Prism software. For more than one treatment groups, post-hoc analysis like Tukey’s multiple comparisons test was also carried out to test for significance. Values of the same have been expressed as F (DFn, DFd). P values were considered significant when they were ≤ 0.05.

5.3 Results

5.3.1 NU9056 and CID 16020046 were both able to reduce alcohol-induced MCP-2 production. As the results from the arrays pointed towards MCP-2 as an inflammatory chemokine which may be positively regulated by H4K12ac, it was further studied to confirm if it was associated with GPR55 and if NU9056 and CID could reversed or inhibit the effects of EtOH. Post-treatment, MCP-2 expression was studied by single-cell imaging flow cytometry for untreated or control MDDCs and MDDCs treated with 0.2% EtOH, NU9056 50nM, CID 5uM or combination of NU9056 and CID with EtOH. Results as shown in figure 11, panel a, MDDCs treated with 0.2% EtOH have significantly increased percentage of cells expressing MCP-2 (76.0% ± 5.06, p=0.0005) compared to untreated control MDDCs (43.3% ± 3.94). Further, MDDCs treated with 0.2% EtOH + NU9056 have significantly decreased percentage of MCP-2 expressing cells (22.2% ± 3.45, p=0.0003) compared to 0.2% EtOH treated MDDCs. Similarly, MDDCs treated with 0.2% EtOH + CID have significantly decreased percentage of MCP-2 expressing cells (18.7% ± 0.57, p=0.0003) compared to 0.2% EtOH treated MDDCs. Panel b show representative overlay histogram of MCP-2 intensity where the rightmost histogram is that of 0.2% EtOH indicating its highest intensity for MCP-2 whereas leftward shift by 0.2% EtOH + NU9056 and 0.2% EtOH + CID show decreased intensity for MCP-2. Panel c shows representative single cell images for each treatment.
5.3.2. Chronic alcohol treatments increase ROS production while NU9056 and CID 16020046 were able to decrease alcohol-induced ROS production. Alcohol has been shown to cause ROS production and oxidative stress (Agudelo et al., 2011, Lu and Cederbaum, 2016, Wang et al., 2015, Liang et al., 2014a). Additionally, inflammation has also been commonly related to oxidative stress and ROS (Dandekar et al., 2015, Satapati et al., 2015). Having shown in our recent publication that H4K12ac may be an inflammation regulator (Parira et al., 2017a), the effect of NU9056 and CID was studied on ROS production by MDDCs to see if H4K12ac associated GPR55 expression had any effect on ROS production by MDDCs. Post treatments, ROS was assayed using DCF-DA and studied by single-cell imaging flow cytometry. As shown in figure 12, panel a, 0.2% EtOH (68.6% ± 8.5, p=0.001) significantly increases percentage of ROS producing cells compared to control MDDCs (45.5% ± 4.5). Additionally, 0.2% EtOH + NU9056 50nM (21.6% ± 3.31, p=0.0001) significantly reduces percentage of ROS producing cells compared to 0.2% EtOH treated MDDCs. Further, 0.2% EtOH + CID 5uM (38.2% ± 14.4, p=0.01) significantly reduces percentage of ROS producing cells compared to 0.2% EtOH treated MDDCs. Panel b shows representative single cell images. Panel c, d and e show representative overlay histograms for different treatments.

5.3.3. NU9056 increases the percentage of cells expressing HLA-DR. To understand whether chronic alcohol treatment and H4K12ac associated GPR55 expression alters dendritic cell antigen presentation functionality, MHC Class II cell surface marker HLA-DR, which has been shown to be expressed by mature dendritic cells that are presenting antigens (Radej et al., 2015, Shariat et al., 2014), was quantified at Day 0 (before starting chronic treatment), at Day 3 and Day 5 of chronic treatment with 0.2% EtOH. At Day 5, HLA-DR was also quantified in MDDCs treated with NU9056 and CID, in the presence or absence of EtOH. As shown in figure 13, panel a, there is no significant difference between
Day 0, 3 or 5 of chronic treatment between control and 0.2% EtOH treated MDDCs. Post chronic treatment, as shown in panel b, NU9056 (87.1% ± 5.5, p=0.05) significantly increases the percentage of cells expressing HLA-DR compared to control or untreated MDDCs (62.2% ± 9.2). All other treatments had no significant differences.

5.3.4. Alcohol’s effect on H4K12ac associated GPR55 expression does not modulate cyclic AMP concentration in MDDCs. To further understand the cell signaling mechanism underlying alcohol-induced H4K12ac associated GPR55 increased expression, cyclic AMP levels were measured in chronically treated MDDCs under three different conditions. Post chronic treatment, MDDCs were divided into three groups, they were either treated with cyclic AMP inducer, Forskolin, or they were treated with beta 2 adrenergic receptor inducer Albuterol or they were left untreated. Cyclic AMP was measured in cell lysates. As shown in the figure 14, even though no significant differences were found, overall, Albuterol failed to increase cyclic AMP in MDDCs treated with alcohol. Hence beta 2 adrenergic receptors do not play a role when it comes to alcohol modulation of the cyclic AMP in MDDCs. Forskolin, however, was able to increase overall levels of cyclic AMP in all treatments except GPR55 agonist 250 nM O1602 treated MDDCs. Concentration of O1602 was determined by XTT viability assay and assaying GPR55 expression (Supplementary figure S2). Further, NU9056 and CID have a similar effect on cyclic AMP levels. These data revealed that even though alcohol showed alterations in cyclic AMP other downstream signaling molecules of GPCRs may be of more importance.

5.4 Discussion

Inflammation and oxidative stress are two important aspects of the pathophysiology of AUDs (Gonzalez-Reimers et al., 2014). Based on data suggesting H4K12ac may be positively regulating MCP-2, we measured MCP-2 levels in MDDCs
treated with NU9056, CID alone or in combination with 0.2 % EtOH. Interestingly, both NU9056 and CID in combination with 0.2% EtOH reduced MCP-2 levels in MDDCs compared to 0.2% EtOH alone. Hence, Both NU9056 and CID could serve as anti-inflammatory drug targets given that they can reduce levels of inflammatory chemokine MCP-2 (Asano et al., 2015). However, we previously saw NU9056 also has a pro-inflammatory effect where it was increasing levels of inflammatory cytokines and chemokines. This needs to be further addressed in the future and levels of these cytokines should be measured after treating MDDCs with CID to understand the role of GPR55 in their expression. To further study the role of H4K12ac as an oxidative stress regulator, ROS levels were measured in MDDCs post chronic treatment. Remarkably, both NU9056 and CID were able to reduce ROS levels compared to 0.2% EtOH; therefore, indicating, firstly, H4K12ac may be an oxidative stress regulator, secondly, both NU9056 and CID may be potential therapeutic antioxidant targets. Previous studies have also shown therapeutic effects of NU9056 and CID. In a mice model of colitis or intestinal inflammation, CID was shown to reduce intestinal inflammation by decreasing macrophage migration and activation (Stancic et al., 2015). NU9056 was also found to have an anti-inflammatory activity where it was found to reduce cytosolic phospholipase A2 which leads to expression or pro-inflammatory factors like eicosanoids, in neuroblastoma cells under oxygen-glucose deprivation injury (Tan et al., 2016b). Based on these studies, our results show NU9056 and CID may be considered as therapeutic drug targets and developed as effective anti-inflammatory or anti-oxidants.

Dendritic cells are one of the primary professional antigen presenting cells (APC’s) (Banchereau et al., 2000) and they are characterized by MHC class molecules on their surface which helps present antigens (Paul and Neefjes, 2013). HLA-DR is one such MHC
class II group molecule that is expressed by mature dendritic cells and help in antigen presentation (Buttari et al., 2008). Apart from being inflammation and oxidative stress regulator, to assess H4K12ac’s role in modulating DC functionality, HLA-DR expression was measured in MDDCs chronically treated with 0.2% EtOH along with MDDCs treated only with NU9056 or CID or 0.2% EtOH + NU9056 and 0.2% EtOH + CID. Results showed the only treatment which had a significant effect was NU9056, significantly increasing HLA-DR compared to untreated MDDCs. This means, blocking H4K12ac alters MDDC function. Previous studies have shown that both acute and chronic alcohol prevents human MDDCs from maturing and differentiating (Buttari et al., 2008). In this study Buttari et al., demonstrated that MDDCs from alcoholics showed decreased CD86 expression and increased HLA-DR expression compared to MDDCs from non-drinkers. Other treatments, however, did not affect HLA-DR expression. Even though previous studies show alcohol modulates HLA-DR, our results of increased maturation or differentiation when H4K12ac is blocked may signify the role of H4K12ac as a modulator of genes responsible for antigen presentation. However, further studies, especially co-localization studies to understand antigen presentation is required to grasp this mechanism in-depth.

Finally, to assess the role of H4K12ac in GPR55 functionality, GPCR downstream signaling molecule, cyclic AMP, (Hill et al., 2010) was measured in MDDCs under chronic alcohol treatment. Apart from measuring baseline levels, post chronic treatment, MDDCs were treated with two different compounds, Forskolin and Albuterol. Interestingly there were no significant differences between any treatments in either group. Albuterol, a beta 2 adrenergic receptor agonist (Luchowska et al., 2009) was not able to increase cyclic AMP levels above baseline, indicating they are not responsible for cyclic AMP being detected in the cells. Forskolin, apart from being able to increase cyclic AMP levels overall
compared to baseline, showed opposite effects when compared between GPR55 agonist O1602 and GPR55 antagonist CID. In addition, NU9056 and CID as expected had the same effect on cyclic AMP compared to control. According to previous studies, GPR55 agonists like lysophosphatidylcholine (LPC) and abnormal Cannabidiol (abn-CBD) did not show any significant modulation in the cyclic AMP (Ruz-Maldonado et al., 2018, Drzazga, 2018 #418); however, CB2 agonists have been shown to inhibit cyclic AMP (Herring et al., 1998). Additionally, literature shows, GPR55 heterodimerizes with CB2 to modulate downstream cell signaling (Balenga et al., 2014), further, GPR55 ligands also promote receptor coupling to multiple cell signaling pathways (Henstridge et al., 2010). Based on these studies, it may be more complex to understand the mechanisms behind H4K12ac-GPR55 signaling and it may be helpful to study other GPCR cell signaling components like calcium levels and NFkB levels (El Zein et al., 2007).
Figure 11: NU9056 and CID 16020046 were both able to reduce alcohol-induced MCP-2 production.

Post chronic treatment, MCP-2 expression was studied by single-cell imaging flow cytometry for untreated or control MDDCs and MDDCs treated with 0.2% EtOH, NU9056 50nM, CID 5uM or combination of NU9056 and CID with EtOH. MDDCs were labelled with eFluor 450 to acquire live cell images only followed by FITC labelled antibody against MCP-2. Panel a shows percentage of FITC positive or MCP-2 expressing single cells. MDDCs treated with 0.2% EtOH have significantly increased percentage of cells expressing MCP-2 (76.0% ± 5.06, p=0.0005) compared to untreated control MDDCs (43.3% ± 3.94). Further, MDDCs treated with 0.2% EtOH + NU9056 have significantly decreased percentage of MCP-2 expressing cells (22.2% ± 3.45, p=0.0003) compared to 0.2% EtOH treated MDDCs. Similarly, MDDCs treated with 0.2% EtOH + CID have significantly decreased percentage of MCP-2 expressing cells (18.7% ± 0.57, p=0.0003) compared to 0.2% EtOH treated MDDCs. 1-way ANOVA showed significant difference between treatments $F(5,36)=37.08, p<0.0001$. The experiment was carried out at least thrice. Data is represented as Mean ± SEM. Statistical differences were calculated using student’s t-test when each treated sample were individually compared to untreated control and 1-way ANOVA followed by Tukey’s multiple comparisons test to test for significance between treatments, significant differences are indicated with * for $p \leq 0.05$. Panel b show representative overlay histogram of MCP-2 intensity. Panel c shows representative single cell images for each treatment.
Figure 12: Chronic alcohol treatments increase ROS production while NU9056 and CID 16020046 were able to decrease alcohol-induced ROS production.

Post chronic treatment, MDDCs were re-treated with NU9056 or CID followed by incubation with 10uM DCF-DA. Post incubation with DCF-DA, for positive control, hydrogen peroxide (50µM H₂O₂) was added, MDDCs receiving EtOH were re-treated with 0.2% EtOH and the untreated control cells received just media. ROS was measured through single-cell imaging flow cytometry after 15 minutes, where post alcohol or H₂O₂ treatment, viability dye DAPI was added to the cells, and 10,000 live single cell images per sample were acquired using Amnis FlowSight. Data were analyzed using Ideas software. DCF-DA positive cells were gated on total alive single cell images. Panel a, 0.2% EtOH (68.6% ± 8.5, p=0.001) significantly increases percentage of ROS producing cells compared to control MDDCs (45.5% ± 4.5). Additionally, 0.2% EtOH + NU9056 50nM (21.6% ± 3.31, p=0.0001) significantly reduces percentage of ROS producing cells compared to 0.2% EtOH treated MDDCs. Further, 0.2% EtOH + CID 5uM (38.2% ± 14.4, p=0.01) significantly reduces percentage of ROS producing cells compared to 0.2% EtOH treated MDDCs. 1-way ANOVA showed a significant difference between treatments (F(5,54)=8.916, p<0.0001). The experiment was carried out at least thrice in duplicates. Data is represented as Mean ± SEM. Statistical differences were calculated using student’s t-test when each treated samples were individually compared to untreated control and 1-way ANOVA followed by Tukey’s multiple comparisons test to test for significance between treatments, significant differences are indicated with * for p≤0.05. Panel b shows representative single cell images. Panel c, d and e show representative overlay histograms for different treatments.
Figure 13: NU9056 increases the percentage of cells expressing HLA-DR.

Post differentiation, HLA-DR expression was quantified by single cell imaging flow cytometry on Day 0 that is before beginning chronic alcohol treatment, on Day 3 after beginning treatment and on Day 5 after finishing chronic treatment. On day 5, MDDCs chronically treated with NU9056 or CID or in combination with EtOH 0.2% was also analyzed for HLA-DR expression. MDDCs were surface labelled with anti-HLA-DR FITC labelled antibody followed by addition of DAPI to gate on live single cell images. As shown in panel a, there is no significant difference between Day 0, 3 or 5 of chronic treatment between control and 0.2% EtOH treated MDDCs. Post chronic treatment, as shown in panel b, NU9056 (87.1% ± 5.5, p=0.05) significantly increases the percentage of cells expressing HLA-DR compared to control or untreated MDDCs (62.2% ± 9.2). All other treatments had no significant differences. The experiment was carried out at least thrice. One-way ANOVA was carried out to check for interaction and did not show any significance. Data is represented as Mean ± SEM. Statistical differences were calculated using student’s t-test when individually compared to untreated control and 1-way ANOVA followed by Tukey’s multiple comparisons test to test for significance between treatments, significant differences are indicated with * for p≤0.05. Panel c shows representative single cell images. Panel d and e show representative overlay histogram of HLA-DR intensity corresponding to panel a and b respectively.
Figure 14: Alcohol’s effect on H4K12ac associated GPR55 expression does not modulate cyclic AMP concentration in MDDCs.

Post chronic treatment, MDDCs were harvested and divided into three groups, one receiving Forskolin, one receiving albuterol and one not receiving any further treatment. Cell lysates from these MDDCs were analyzed for cyclic AMP levels. No significant differences were found. Data is represented as Mean ± SEM from at least two experiments. Statistical differences were calculated using student’s t-test when individually compared to untreated control and 1-way ANOVA followed by Tukey’s multiple comparisons test to test for significance between treatments.
Supplementary figure S2: XTT and GPR55 expression measurement assay point towards O1602 250nM as optimum concentration for chronic treatments. Panel a: XTT assay shows 5uM O1602 (37% ± 3, p<0.0001) and 10uM O1602 (34% ± 1, p<0.0001) causes significant cell death compared to untreated MDDCs. Other treatments do not have any significant difference. Experiment was carried out at least twice. Data is represented as Mean ± SEM. Statistical differences were calculated using student’s t-test when individually compared to untreated control and significant differences are indicated with * for p≤0.05. GPR55 expression measurement by single cell imaging flow cytometry showed 250 nM O1602 to cause maximum increase in expression of GPR55 compared to untreated control. Panel b shows percentage of GPR55 positive single cells in MDDCs treated with O1602, concentration ranging from 25nM to 1uM. Panel c shows mean intensity of GPR55 representative of one experiment.
CHAPTER 6: Pitfalls and Alternative Strategies as future directions for this project

This project overall was successful in elucidating the changes in epigenetic landscape particularly H3 and H4 quantity and H3 and H4 post-translational modifications using an in vitro model of chronic alcohol exposure of MDDCs. It was also able to show a correlational effect between H4K12ac and cannabinoid receptor GPR55 expression. Finally, it was also able to demonstrate a partial anti-inflammatory effect of NU9056 and CID suggesting H4K12ac be an inflammation regulator. It was also able to demonstrate the partial anti-oxidative property of NU9056 and CID suggesting H4K12ac to be an oxidative stress regulator in addition to an inflammation regulator.

There were, however, some pitfalls that the project saw that could be overcome by utilizing alternate strategies. Some of these pitfalls have been discussed below.

a. In the study to understand the purity of dendritic cell population and the effect on the same due to chronic culture with alcohol, about 10% of cell population was CD11c and CD14 double negative cells, about 10% was purely monocytic or CD14 positive cells. These two groups which in total about 20% of cell population consist of cells other than dendritic cells may give rise to certain discrepancies in the studies. However, the other 80% of cell population is CD11c positive; hence, dendritic cells in either early or late stages of differentiation. This may contribute towards some understanding based on results as purely homogenous cell populations may give slightly different results than what we have observed (Aldo et al., 2013). This may be overcome by sorting cell populations after monocyte differentiation and prior to alcohol treatments to only carry out experiments in homogeneous cell populations.
b. The second pitfall in the project lies in the utilization of NU9056 to block H4K12ac. Even though we showed for the first time the ability of NU9056 to bring alcohol-induced H4K12ac down to basal levels, it was not the most specific method to obtain the result. We observe this in the inflammatory cytokine arrays, where we saw a further increase in several cytokines and chemokines that were upregulated by alcohol apart from the decrease we saw in the alcohol-induced MCP-2. This may have happened due to several reasons including drug-drug interactions with alcohol, lack of specificity of NU9056 for TIP60 and finally the selected non-toxic concentration of NU9056 may not be enough to block TIP60 completely. As an alternative strategy, more specific siRNA or crispr-cas9 based silencing or knockdown of TIP60 gene may have a more pronounced effect on H4K12ac helping us better understand its functionality. We have recently published siRNA-based silencing of TIP60 (Castillo-Chabeco et al., 2018); however, these experiments were performed in cells treated acutely with alcohol. Therefore, we need to carry out those experiments after exposure to different concentrations of alcohol and kinetic studies.

c. The final pitfall lies in the question of whether alcohol itself or its metabolites have a role to play in the epigenetic effects as seen under chronic alcohol effects. To explore this question, we carried out an experiment where we pretreated MDDCs with alcohol dehydrogenase enzyme inhibitor 4 Methylpyrazole or 4-MP followed by chronic alcohol treatment. Post-treatment, we measured H4K12ac in MDDCs treated with 4-MP, 0.2% EtOH or both using single cell imaging flow cytometry. Results as shown in Supplementary figure S3, as expected 0.2% EtOH (85.67% ± 5.7, p=0.0078) upregulated percentage of H4K12ac positive cells compared to untreated MDDCs (64.1% ± 1.8). 4-MP by itself does not affect the percentage of
H4K12ac positive cells compared to control; however, in combination with 0.2% EtOH (70% ± 3.6), it reduced the percentage of H4K12ac positive cells compared to 0.2% EtOH. These data indicate alcohol metabolites may have a role to play towards the epigenetic effects that we see after alcohol exposure. However, this may also be a result of toxicity caused by 4-MP. Previous literature show that 4-MP may cause toxicity in rats and mice after chronic alcohol consumption (Magnusson et al., 1972, Kager and Ericsson, 1974). Even in recent reports, 4-MP has been shown to cause increased DNA adduct formations and oxidative stress (Sachse et al., 2016). Hence the effects of 4-MP that we see may stem from toxicity. To further confirm this as a future direction to this aspect, viability and toxicity assays using 4-MP and other alcohol metabolites including assays for apoptosis and necrosis to rule out toxicity as a confounding factor must be carried out.
Supplementary Figure S3: Role of alcohol dehydrogenase inhibitor in alcohol induced H4K12ac.

MDDCs were pretreated with alcohol dehydrogenase enzyme inhibitor 4 Methylpyrazole or 4-MP followed by chronic alcohol treatment. Post-treatment, H4K12ac was measured in MDDCs treated with 4-MP, 0.2% EtOH or both using single cell imaging flow cytometry. As shown in the upper panel, 0.2% EtOH (85.67% ± 5.7, p=0.0078) upregulated percentage of H4K12ac positive cells compared to untreated MDDCs (64.1% ± 1.8). 4-MP by itself does not affect the percentage of H4K12ac positive cells compared to control, however, in combination with 0.2% EtOH (70% ± 3.6), it reduced the percentage of H4K12ac positive cells compared to 0.2% EtOH but not significantly. 2-way ANOVA showed a significant column factor between treatments (F(4,20)=68.5, p<0.0001). The lower panel shows representative images. The experiment was carried out from four buffy coats. Data is represented as Mean ± SEM. Statistical differences were calculated using 2-way ANOVA followed by Tukey’s multiple comparisons test to test for significance between treatments, significant differences are indicated with * for p≤0.05.
Chapter 7: CONCLUSION

7.1 Conclusions

A complex disorder like AUD with adverse outcomes ranging from that of injuries due to inebriation to liver cirrhosis and cancer needs all the support form researchers to overcome this formidable cause of death. The starting point of therapy lies in understanding the causes and mechanisms of a disorder or disease. Even after years of research on AUDs, the exact signaling mechanism that alcohol uses to cause its effects are unknown. Epigenetics, a study of all those factors that modulate gene expression may contain the answer to this question. The purpose of this project was to understand the underlying epigenetic mechanism to alcohol's effect in human MDDCs. Results from this project were able to reveal that chronic alcohol treatments increase acetylation at H4K12 in human MDDCs treated in vitro with alcohol. In order to understand how chronic alcohol exposure was able to increase acetylation at a histone residue we have to go back to the basics of mechanism of histone modifications and chromatin remodeling. Chromatin is packaged in nucleosomes inside a cell nucleus for it to be accommodated inside the nucleus and regulate gene transcription and translation (Carone et al., 2014). Nucleosomes is a very effective way to regulate transcription as it either includes or occludes promoters from being exposed to transcription factors from binding to regulate gene expression (Lorch and Kornberg, 2015). This is where chromatin remodelers come into the picture (Clapier and Cairns, 2009). Remodelers or chromatin remodeling complexes use energy from ATP hydrolysis to remodel chromosome that includes moving, destabilizing, ejecting, or restructuring nucleosomes in the chromatin (Clapier and Cairns, 2009). There are various families of remodelers, the SWI/SNF (switching defective/sucrose nonfermenting) family remodelers, ISWI (imitation switch) family remodelers, CHD (chromodomain, helicase, DNA binding) family remodelers and INO80
(inositol requiring 80) family remodelers as reviewed by Clapier et al. (Clapier and Cairns, 2009). Further, histone modification enzymes like the HDACs, HATs and KMTs can act on nucleosomes to bring about post-translational modifications (Gong and Miller, 2013). Hence in order for a substance to modulate epigenetic modifications, it may be directly affecting the remodelers or the histone modification enzymes or a combination of both. Not much literature exists in the effect of alcohol on chromatin remodelers but recently a group of researchers found that BAF (BRG1/BRM-Associated Factor) part of the SWI/SNF group of remodeler showed ethanol sensitivity in neural progenitor stem cells (Burrowes et al., 2017). Another article studied alcohol effects in Caenorhabditis elegans and found SWI/SNF chromatin remodeling regulates alcohol response behaviors in the same (Mathies et al., 2015). However, more studies need to be carried out to see the effects of alcohol on chromatin remodelers to further understand the mechanism of alcohol’s effect on specific histone modifications. A lot of studies do exist on alcohol’s effect on histone modifying enzymes as discussed previously, which may be another method by which it is bringing about its epigenetic effects.

Chronic alcohol treatments also modulate functionality of human MDDCs by upregulating GPR55 gene and protein, modulating inflammation via cytokine and chemokine secretion, oxidative stress via ROS production, and cell surface markers. All of these processes get modulated when H4K12ac is inhibited. To understand how H4K12ac can modulate such functions in MDDCs we have to take a closer look at the basic function of a histone acetylation. Histone acetylation in general is known to open up chromatin and make it available to transcription based on the negative charge of the histone acetyl groups (Gräff and Tsai, 2013). Acetylated lysines have been shown to be recognized by bromodomain containing proteins (Marmorstein and Zhou, 2014). Hence, when we call H4K12ac as an inflammation and oxidative stress regulator, what it might
mean is that certain bromodomain containing protein like bromodomain-PHD finger protein 1 (BRPF1) subunit of the monocytic leukemia zinc (MOZ) histone acetyltransferase (HAT) complex may bind to H4K12ac (Lubula et al., 2014) and lead to availability or promoters of genes responsible for inflammation, oxidative stress or even cell surface receptor expression. Once promoters are available, transcription factors along with RNA polymerase II can bind to promoters which leads to gene expression (Stasevich et al., 2014). Certain groups of transcription factors are important players in inflammation like HIF (hypoxia-inducible factor) (Palazon et al., 2014), Egr2 and Egr 3 in B and T cells (Li et al., 2012), MEF2c, NFκB and KLF2 (Xu et al., 2015). The study of these transcription factors, may help further understanding the role of alcohol induced H4k12ac in regulating inflammation and oxidative stress. In conclusion, this project touches the surface of alcohol induced epigenetic modifications with emphasis in a small subset of cells and specific histone modifications revealed to be significantly upregulated after alcohol treatment in vitro. Therefore, further studies are necessary to understand this mechanism in-depth and to translate our findings using cells or biological samples from alcohol users.

7.2 Future Directions

Future directions for this project has some open ended questions for further research such as,

- What is the histone acetylation state of H4K12 at the promoter region of GPR55?
- What is the histone acetylation state (H3 and H4 acetylation) at gene and promoter regions of CB1, CB2 and GPR55?
- What downstream signaling molecules does this alcohol employ to bring about inflammatory and oxidative stress effects?
- Finally, will more specific blocking of H4K12ac and GPR55 help understand the significance of this pathway towards finding effective therapeutic treatment for AUDs?
- Since all experiments were carried out in vitro after treatment of commercially available cells, as a future direction these findings should be translated into ex-vivo experiments from alcohol users at different stages of AUDs.
CHAPTER 8: SIGNIFICANCE

The significance of this study lies in elucidating, for the first time, a novel epigenetic alteration observed in human MDDCs under the effect of chronic alcohol exposure. H4K12ac may have a significant role as an epigenetic biomarker and in the future may be developed for diagnostic purposes. Further, this study also elucidates, for the first time, an association between H4K12ac and cannabinoid receptor GPR55 expression under chronic alcohol exposure. This possible mechanism of alcohol action opens up several opportunities to understand the same in-depth. Additionally, this project also sheds light on two different possible therapeutic drug targets, NU9056 and CID, both of which compounds were able to show partial anti-inflammatory and anti-oxidative effects in alcohol treated MDDCs. However, further pharmacology based experiments both in vitro and in vivo are necessary to draw conclusions about their effectiveness as therapeutic drugs. In conclusion, the project was able to achieve its major goal of identifying a novel epigenetic pathway underlying alcohol effects in human MDDCs treated in vitro with chronic alcohol levels.
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