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Impact of Alcohol on Intestinal Homeostasis

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DEDICATION

This dissertation is dedicated to my **Grammy and Poppop.** I love you both so much! Thank you for the emotional, spiritual, and financial support throughout my graduate school years. I couldn't have done it without you.

ABSTRACT OF THE DISSERTATION

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by

Tasha Marie Barr

Doctor of Philosophy, Graduate Program in Biomedical Sciences

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Dr. Ilhem Messaoudi, Co-Chairperson

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Chronic heavy alcohol consumption results in intestinal injury characterized by increased permeability, dysbiosis, nutrient malabsorption, increased risk of colorectal cancer, and functional alterations in mucosal immune cells. Studies have implicated dysregulation of tight junction proteins as well as quantitative and qualitative changes in gut microbiota as key contributors to gut injury. Recently, we showed using a macaque model of voluntary ethanol self-administration that moderate consumption resulted in a more robust T-cell and antibody vaccine response to Modified Vaccinia Ankara (MVA), while heavy drinkers generated blunted T-cell and antibody response compared to controls. First, we show that chronic alcohol consumption modulates host defense and response to booster vaccination by altering gene expression in circulating leukocytes. Using this model, we

previously examined the effect of chronic ethanol consumption on cytokine production using intracellular cytokine staining, and found that colonic T cells exhibited a disruption of inflammatory cytokine production. Next, we show the modulation of transcriptome and microbiota profiles by chronic alcohol consumption within the different segments of the gastrointestinal tract. Lastly, we report alterations in the transcriptional profiles of intestinal lamina propria lymphocytes following chronic alcohol consumption. Since our animals lack overt organ damage, the alterations in gene expression and microbial composition precede clinical disease and could potentially serve as early biomarkers before detrimental effects occur. These findings provide novel insight into the mechanisms of ethanol-induced mucosal damage and lay the foundation for follow up studies to develop new interventions to reverse mucosal damage and improve immunity in heavy alcohol abusers.

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Chapter 1: Introduction and review of literature

1.1 Alcohol use disorder

The National Institute on Alcoholism and Alcohol Abuse (NIAAA) defines alcohol use disorder (AUD) as a chronic relapsing disease characterized by an impaired ability to stop or control alcohol use despite adverse consequences. The Substance Abuse and Mental Health Services Administration (SAMHSA) further defines AUD as binge drinking on 5 or more days in the past month. Binge drinking is a pattern of drinking that brings blood alcohol concentration (BAC) levels to 80 mg/dL (~4 drinks for women and ~5 drinks for men) in about 2 hours. Almost 18 million individuals in the United States meet the criteria for AUD, which results in 88,000 deaths [1, 2] and $223.5 billion in health care costs annually [3]. On a global scale, AUD is the third leading risk factor for poor health globally, and is responsible for 2.5 million deaths, including 320,000 young people between 15 and 29 years of age annually [4].

It is well established that AUD leads to significant organ damage and increased susceptibility to bacterial and viral infections, notably bacterial pneumonia [5, 6], tuberculosis [7-9], hepatitis C virus (HCV) [10, 11], and human immunodeficiency virus (HIV) [12]. Additionally, AUD disrupts the intestinal barrier resulting in the translocation of gut-derived endotoxins causing inflammatory responses in the liver that contribute to

alcoholic liver disease (ALD). AUD is also associated with an increased risk for developing liver, head and neck, and colorectal cancers [4, 13-15].

Alcoholic beverages are energy dense and often become the primary energy source in those with AUD, consequently these individuals maintain body weight while suffering from malnutrition. Individuals with AUD are often deficient in one or more essential nutrients including vitamin A, vitamin C, vitamin D, vitamin E, folate, and thiamine [16]. Amongst a group of 30 male and female patients who consumed at least 100g of ethanol daily (on average 51% of total caloric intake) for at least 5 years, all subjects were deficient in vitamin E and folate, 83% were deficient in vitamin C, 80% were deficient in vitamin A, and 73% were deficient in thiamine [17]. Acetaldehyde, the most toxic metabolite produced, is able to degrade folate, which could contribute to this deficiency. Folate deficiency has also been associated with colon cancer [18]. Reduced levels of vitamin D can lead to bone weaknesses. Deficiencies in antioxidant vitamins such as vitamin C and vitamin E exacerbate oxidative damage. Epidemiological studies have associated low levels of various vitamins and minerals with hypertension, cardiovascular disease, stroke, and cancer [18]. These micronutrients play an important role in immune system homeostasis and barrier integrity as discussed later in this introduction [19].

The impact of moderate/non-heavy alcohol consumption is largely understudied despite the fact it is widespread [20]. WHO estimates approximately 1/3 of the world population moderately consumes alcohol. According to the 2015-2020 Dietary Guidelines for Americans, moderate alcohol consumption is up to 1 drink/day for women and up to 2 drinks/day for men. Large epidemiological studies show that moderate alcohol

consumption may have health benefits including decreased risk for heart disease, ischemic stroke, and diabetes [21]. Several studies have described a J-shaped curve for the effects of alcohol on human health, with light to moderate consumers having a lower risk of all-cause mortality than abstainers, while those with AUD are at the highest risk [22].

1.2 Alcohol metabolism

Alcohol is primarily metabolized in the stomach, small intestine, and liver by alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP2E1; Fig. 1.1) [23]. ADH is present in the cytosol whereas CYP2E1 is present predominantly in microsomes. Both enzymes convert alcohol to acetaldehyde, which is further metabolized to acetate by acetaldehyde dehydrogenase (ALDH) in the mitochondria. Acetate is then released into the blood where it is oxidized to carbon dioxide in the heart, skeletal muscle, and brain [23].

Acetaldehyde is the toxic byproduct that contributes to tissue damage and alcohol dependence [23]. It can also bind to other proteins to form adducts, such as malondialdehyde (MDA) and MDA-acetaldehyde (MAA), which play a key role in the development of liver injury and stimulate antibody responses that further promote liver inflammation and fibrosis [24]. In addition, oxidation of ethanol by CYP2E1 leads to the formation of reactive oxygen species (ROS). Elevated levels of ROS cause oxidative stress which has been shown to play a role in several harmful processes including cancer development, atherosclerosis, diabetes, and inflammation [24].

CYP2E1 and catalase can also metabolize alcohol in the brain. Catalase is localized to peroxisomes and requires hydrogen peroxide to oxidize alcohol into water and

acetaldehyde. Alcohol metabolism can also take place in the pancreas by acinar and pancreatic stellate cells, which contributes to the development of alcoholic pancreatitis [25]. Additional studies are required to fully understand the role of ethanol metabolites and adducts in the development of alcoholic liver injury and organ damage.

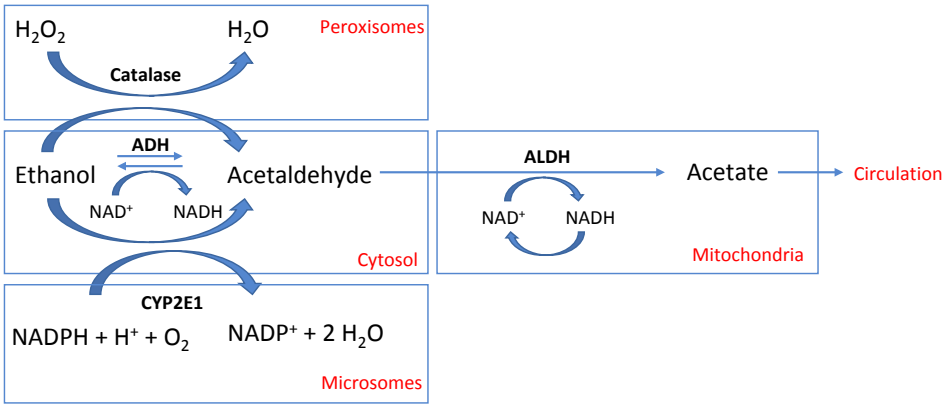


Figure 1.1: Pathways of ethanol metabolism. Biochemical pathways of ethanol metabolism.

1.3 Animal models used in alcohol research

There are both ethical and experimental concerns associated with conducting alcohol research exclusively in humans. Such studies can be challenging because of difficulties in obtaining accurate medical histories, collecting longitudinal samples, as well as confounding factors such as diet, sleep-wake cycles, homelessness, and other drug use [26]. Moreover, it is unknown whether the malnutrition and nutrient deficiencies observed in alcoholics are primarily due to poor lifestyle habits or alcohol-mediated disruptions in the absorption and assimilation of dietary micronutrients [27].

Rodents are the most commonly used animal models in alcohol research and such studies offer several advantages such as availability of transgenic models that can facilitate mechanistic studies. However, rodent models almost exclusively rely on short-term heavy and binge ethanol intake protocols that result in rapid onset of liver pathology. Specifically, rodent models utilize high concentration of ethanol (20% ethanol) in drinking water as the sole source of fluid, a possible stressor in itself. In addition, other rodent models utilize oral gavage as means of ethanol administration. Intestinal epithelial cell lines are also utilized to examine the impact of alcohol on intestinal homeostasis. However, the impact of ethanol consumption on organ systems such as the gut cannot be fully understood by carrying out *in vitro* studies since ethanol metabolites (acetaldehyde, acetate, reactive oxygen species, and lipid peroxidation products) exert a significant physiological impact. These products can interact with other molecules to form adducts which play a critical role in liver and gut injury as well as immune modulation [28-30]. Moreover, epithelial cell

lines do not recapitulate the complex interaction between mucosal epithelial cells, immune cells, and the microbiome.

The rhesus macaque is one of the most commonly used nonhuman primate (NHP) models in biomedical research, sharing 93.54% genetic homology to humans [31]. Additionally, NHPs voluntarily consume with drinking patterns that capture the spectrum seen in humans thereby facilitating studies in an outbred species while maintaining rigorous control over diet and other environmental cues. Moreover, the immune system of rhesus macaques is similar to that of humans and these animals are susceptible to several clinically important pathogens making them a valuable model to study the impact of ethanol on immunity [32]. Nonetheless, NHP models come with their disadvantages as well. Costly requirements such as dedicated facilities to house the animals, experienced personnel to perform specialized procedures, and compliance with high standards of care must be considered.

In the studies presented in this dissertation, we leverage a nonhuman model of voluntary ethanol self-administration developed by Dr. Kathy Grant (Division of Neuroscience, Oregon National Primate Research Center) [33, 34]. This model uses a schedule-induced polydipsia to establish reliable ethanol consumption in rhesus macaques under open access conditions and allows natural segregation of non-heavy (moderate) and heavy drinkers [33, 34]. Following a four month induction period, animals are given "open-access" to 4% ethanol or water for 22 hours/day resulting in ~35% of macaques becoming heavy drinkers as defined by daily ethanol intakes that exceed 3g/kg/day (>12 drinks equivalent in humans). Patterns of ethanol consumption are typically established after 3

months of self-administration and are maintained for the duration of the study [33]. Co-housed control animals are given a calorically matched daily maltose-dextrose solution.

1.4 Overview of the immune system

The immune system is an intricate network of molecules, cells, tissues, and organs that work together to defend the body against infectious agents and malignant cells. It is broadly divided into innate and adaptive branches, which are both essential for efficient host defense. The innate immune response is immediately available, but is not specific for any one pathogen. Innate immune cells such as natural killer (NK) cells, neutrophils, monocytes/macrophages and dendritic cells (DCs) express pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), such as the bacterial cell wall component lipopolysaccharide (LPS). The interaction between PRR and PAMPs activates cells of the innate immune system to engulf pathogenic microbes and to secrete cytokines and chemokines, resulting in the induction of an inflammatory response and mobilization of immune cells into the site of infection. In addition, DCs and monocytes process and present peptides derived from foreign antigens bound to specialized molecules called major histocompatibility complex (MHC) molecules on their cell surface to naïve T lymphocytes thereby activating them and initiating the adaptive immune response [35].

The adaptive immune system can be subdivided into cell-mediated immunity, carried out by T cells, and humoral immunity, carried out by B cells. T cells expressing the CD4 T cell co-receptor are known as T helper cells and play a critical role in the activation and maturation of monocytes, cytotoxic T cells and B cells. T cells expressing the CD8 T cell

co-receptor are known as cytotoxic T cells and eliminate host cells infected with intracellular pathogens as well as tumor cells. B cells mature into plasma cells that produce antibodies, also known as immunoglobulins (Ig), to eliminate extracellular microorganisms and prevent the spread of infection. The adaptive immune response can be distinguished from innate immunity by the capability of generating immunological memory, or protective immunity against recurring disease caused by the same pathogen [35].

1.5 Opposing effects of alcohol on the peripheral immune system

It is known that alcohol consumption alters both innate and adaptive immunity in both humans as well as animal models, however these effects have not been systematically assessed on the basis of the amount and duration of alcohol consumption.

1.5.1 Modulation of innate immunity by alcohol

Ethanol modulates the function of monocytes, immature innate immune cells that circulate in the blood until recruited into tissues, in a dose and time dependent manner. Monocytes, which have a lifespan of approximately of 3 days but can survive for weeks in culture [36], express Toll-like receptor (TLR) 4, which is the PRR responsible for recognizing the endotoxin LPS on the surface of Gram negative bacteria [35]. TLR2, along with heterodimer partners TLR1 and TLR6, as well as TLR5 recognize other bacterial products such as triacyl and diacyl lipoproteins, and flagellin. TLR3, TLR7, TLR8, TLR9, TLR11, TLR12, and TLR13 are expressed on intracellular membranes due to their critical nucleic acid-sensing roles [37]. Upon LPS binding, monocytes become activated, mature

into macrophages and migrate into tissues where they respond to infection by secreting various cytokines, recruiting additional leukocytes via production of chemokines and presenting pathogen-derived peptides to T cells to activate them. These events depend on the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) heterodimer p50-p65 and its translocation to the nucleus leading to the expression and production of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor (TNF)-α [35]. Often, investigators stimulate with LPS after pre-exposure to ethanol to mimic inflammation observed in trauma patients with high blood alcohol levels to explore the alterations in immunity that lead to frequent subsequent infections among this group.

Several studies have shown a dose-dependent effect of alcohol on the LPS response by monocytes/macrophages. Pre-incubation of human monocytes isolated from healthy men and women *in vitro* with 25mM alcohol [~0.1g/dL blood alcohol concentration (BAC)] for 24 hours inhibits nuclear translocation of NFκB in response to LPS, and thereby production of pro-inflammatory cytokines [38]. I*n vitro* exposure of the macrophage cell line RAW 264.7 and human peripheral blood monocytes to 25mM ethanol for 24 hours followed by stimulation of LPS also leads to decreased TNF-α production by increasing the expression of IL-1R-associated kinase-monocyte (IRAK-M), a negative regulator of LPS signaling [39]. Similarly, exposure of the human monocytic cell line mono Mac 6 cells to 25mM, 50mM, or 75mM ethanol for 24 hours inhibited LPS and phorbol myristate acetate- (PMA) induced TNF-α production in a dose-dependent manner [40]. This inhibitory effect on NFκB activity is partly due to the increased proteolytic degradation of

IκBα kinase (IKK) and consequent decreased phosphorylation of the NFκB p65 subunit [41]. Additional studies also showed that exposure of RAW 264.7 macrophages and human peripheral blood monocytes to 25mM of ethanol for as little as 60 minutes results in the activation of the heat shock transcription factor-1 (HSF-1), which in turn induces heat shock protein hsp70 expression [42]. Hsp70 binds the NFκB subunit p50 and decreases its nuclear translocation while HSF-1 binds to the TNF-α promoter region resulting in negative regulation of TLR4 signaling [38, 42]. Finally, *in vitro* exposure of human peripheral blood monocytes to 25mM ethanol for 6 hours also inhibited TLR8-induced production of the pro-inflammatory cytokine TNF-α and increased production of the anti-inflammatory cytokine IL-10 [43].

These *in vitro* results have been recapitulated *in vivo* in rodent models. Measurement of serum cytokine levels 2 hours following a one-time administration of ethanol at 6g/kg body weight by oral gavage in female mice (a murine model of binge drinking that yields a peak BAC of approximately 0.4%, which results in loss of consciousness in humans) showed decreased production of inflammatory cytokines IL-6 and IL-12 in response to TLR2/TLR6 (zymosan A *Saccharomyces cerevisiae*), TLR4 (LPS), TLR5 (bacterial flagellin), TLR7 (R-848), and TLR9 (CpG DNA) ligands administered by intraperitoneal or intravenous injection at the same time as ethanol [44]. In addition, production of IL-10 in response to TLR2/6 stimulation was increased [44]. This same treatment also inhibited the *in vitro* production of IL-6 and IL-12 by peritoneal macrophages harvested 2 hours following injection of LPS [45]. Finally, ethanol administered at 6g/kg but not 3g/kg by oral gavage in mice significantly increased serum

concentrations of positive acute phase proteins amyloid A and P that arise early in the inflammatory response and recruit immune cells to the inflammatory site, indicating that ethanol modulates acute phase response in a dose-dependent manner [46]. This phenomenon was not observed in a TLR4 mutant mouse, indicating that the acute phase response is mediated by TLR4 [46].

Recently, it was reported that a single episode of binge alcohol consumption in alcohol-experienced human volunteers (men and women) initially (within the first 20 min) increased total number of peripheral blood monocytes and LPS-induced TNF-α production when blood alcohol levels were ~130mg/dL. However, similarly to the *in vitro* studies described above, at 2 and 5 hours post-binge the numbers of circulating monocytes were reduced and levels of anti-inflammatory IL-10 levels were increased [47].

In contrast to the inhibitory effects of acute alcohol treatment (up to 24 hours), prolonged exposure of human (men and women) peripheral blood monocytes to 25mM ethanol for 7 days increased LPS-induced TNF-α production without affecting IL-10 production [43]. Prolonged exposure of Mono Mac 6 cell line to 25mM, 50mM and 75mM ethanol for 7 days also reverses the initial inhibition of LPS or PMA-induced TNF-α production in a dose-dependent manner [40]. Studies using the RAW 264.7 macrophage cell line and peripheral blood monocytes isolated from healthy men and women demonstrated that this switch to a pro-inflammatory response occurs via decreasing IRAK-M and increasing IRAK-1 and IKK expression resulting in increased phosphorylation of the NFκB p65 subunit, increased NFκB translocation to the nucleus and greater TNF-α production in response to LPS stimulation [39].

Finally, primary alveolar macrophages isolated from female mice cultured in 25-100mM ethanol for 24 hours prior to addition of apoptotic cells showed a dose-dependent decrease in efferocytosis, the process of clearing dying cells that is critical to resolution of the inflammatory process after infection. This defect was rescued when cultures were treated with the Rho kinase inhibitor, Y27632 indicative that ethanol reduced efferocytosis through the induction of Rho kinase activity in a dose-dependent manner [48]. In addition, female mice that consumed 20% (w/v) ethanol for 8 weeks showed a reduction in LPS activated efferocytosis [48]. In contrast to the effects of high ethanol doses, human monocytes isolated after 30 days of moderate beer consumption (330mL for women and 660mL for men) exhibited increased phagocytic, oxidative burst, and intracellular bactericidal activity when incubated with fluorescence-labeled *E. coli* compared to basal levels [49].

As described above, *in vivo* ethanol ingestion of 6.3% (v/v) in the form of a standard Lieber-DeCarli liquid diet for 4 weeks in male mice up-regulated NFκB activation and increased circulating levels of IL-6 and TNF-α in response to TLR4 ligand LPS stimulation [50]. Male rats on a liquid diet with 35% of calories coming from ethanol also showed enhanced mRNA half-life and protein expression of LPS-induced TNF-α by increasing TNF-α in liver monocytes/macrophages [51]. In humans, peripheral blood monocytes isolated from 16 hospitalized male patients with alcoholic hepatitis (but no detectable blood alcohol levels at the time of blood collection) had significantly increased TNF-α production in response to LPS stimulation when compared to monocytes from healthy volunteers [52].

The dendritic cell (DC), which plays a critical role in T cell activation and initiation of adaptive immune responses, is another innate immune cell affected by ethanol. DCs uptake antigens in peripheral tissues which leads to their maturation, and then travel to draining lymph nodes where they present them to T cells [35]. As described above for monocytes, long-term *in vitro* treatment of myeloid DCs (mDCs) generated from healthy female and male blood donors with 25mM ethanol for 7 days results in reduced IL-12 production, increased IL-10 production, and a decrease in expression of the co-stimulatory molecules CD80 and CD86 [53]. Similarly, consumption of 10% (w/v) ethanol in tap water *ad libitum* for 2 days in mice resulted in decreased bone marrow DC generation, decreased expression of CD80 and CD86, impaired induction of T cell proliferation, and a decrease in IL-12 production [54]. Also, monocyte-derived mDCs obtained from healthy male and female volunteers shortly after consuming 2mL vodka/kg body weight in a total volume of 300mL orange juice resulting in BACs of 0.095 ± 0.02g/dL, showed reduced ability to induce T cell proliferation in response to allogeneic antigen, super-antigen staphylococcal enterotoxin B, or tetanus toxoid, indicative of impaired antigen presentation [55].

Finally, in our NHP model of ethanol self-administration, PMA-induced production of the growth factors hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF), and vascular-endothelial growth factor (VEGF) by peripheral blood mononuclear cells (PBMCs) isolated from male and female rhesus macaques after 12 months of chronic ethanol exposure were inhibited [56]. The reduced production of growth factors in this nonhuman primate model of ethanol self-administration was due to increased expression of microRNA miR-181 and miR-221, which led to reduced expression of the

transcription factors, signal transducer and activator of transcription 3 (STAT3) and aryl hydrocarbon receptor nuclear translocator (ARNT) [56].

In summary, several *in vitro* and *in vivo* studies demonstrate that ethanol modulates the function of innate immune cells (monocytes and DCs) in a dose- and time-dependent manner (Table 1.1). Acute high dose exposures inhibit whereas long-term treatments stimulate pro-inflammatory cytokine production. In addition, *in vivo* consumption of moderate amounts enhances phagocytosis and reduces inflammatory cytokine production whereas chronic consumption of large doses inhibits phagocytosis and production of growth factors.

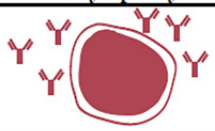
CELL	Moderate	Heavy	
	Chronic	Acute	Chronic
Monocyte 	↑ Phagocytic activity ↑ IL-6, TNF-α	↓ Frequency ↓ IL-6, IL-12, TNF-α ↓ Effercytosis ↑ IL-10	↑↑↑ TNF-α
Dendritic cell 			↓ IL-12 ↑ IL-10 ↓ CD80/CD86
T lymphocyte 	↑ Frequency ↑ IL-2, IL-4, IL-10, IFN-γ ↓ IFN-γ/IL-10 ratio ↑ Vaccine responses	↑ Apoptosis	↓ Frequency ↓ Naive T cells ↑ Memory T cells ↑ Activation ↓ Antigen-specific responses
B lymphocyte 	↑ Frequency ↓ IgA, IgM, IgG	↑ Apoptosis ↑ IgA	↓ Frequency ↑↑↑ IgA, IgM

Table 1.1: Opposing effects of alcohol consumption on immune cells

1.5.2 Modulation of adaptive immunity by alcohol

Alcohol consumption also impacts cell-mediated and humoral immunity. In a very early study, alcohol abuse was associated with a reduction in CD4 and CD8 T cell numbers [57]. This general finding was confirmed in a cross-sectional study of heavy male drinkers (90-249 drinks/month) who displayed lower B cell numbers than moderate (30-89 drinks/month) or light (<10 drinks/month) drinkers [58]. Additionally, 153 chronic alcoholics (men and women) without liver disease had a decreased ratio of CD4/CD8 T cells in peripheral blood [59]. Thus, in humans it appears that alcohol consumption can lower lymphocyte numbers, and decreases are most apparent in those with AUD.

Ethanol consumption by weanling ICR (outbred) mice (adjusted to 6% in their drinking water) for 8 weeks also resulted in 75% fewer CD3+ T cells [60]. Likewise, male rats fed an ethanol-containing liquid diet (8.7% v/v for up to 4 weeks) experienced a progressive loss of both CD4+ and CD8+ T cells [61]. Increased apoptosis of T and B lymphocytes isolated from the thymus, spleen, and lymph nodes of female mice was observed following 16 hour culture with 0.4%-2% ethanol, concentrations 5 to 25 times the definition of intoxication [62]. In contrast to these observations, moderate consumption of beer (330mL for women and 660mL for men) for 30 days resulted in a significant increase in the number of leukocytes, mature CD3+ T lymphocytes, neutrophils and basophils in women, while only basophils were increased in men [63].

In addition to decreased lymphocyte frequency, AUD is also associated with shifts in T lymphocyte phenotype. Decreased percentage of CD45RA+ naïve CD4 and CD8 T cells and an increased percentage of CD45RO+ memory subsets was observed in adult

males who consumed 30.9 ± 18.7 alcoholic drinks/day (400g/day) for approximately 25.6 ± 11.5 years [64, 65]. Similarly in male and female mice, chronic ethanol consumption of 20% ethanol in water for up to 6 months decreased the percentage of naïve T cells and increased the percentage of memory T cells due to increased homeostatic proliferation [66-68]. Accumulation of memory T cells is associated with increased incidence of chronic inflammatory diseases and age-related pathologies such as osteoporosis, sarcopenia, Alzheimer's disease, cancer, and cardiovascular disease [69, 70]. In addition, loss of naïve T cells would be expected to interfere with the development of efficacious responses to infection and vaccination [71].

Alcohol abuse also leads to a significant elevation of activated CD8 T cells, measured by increased expression of human leukocyte antigen (HLA)-DR in adult males who consumed an average of 23 drinks/day for approximately 27 years that persisted for up to 10 days of abstinence [72]. Similarly, an increased percentage of CD8 T cells expressing HLA-DR and CD57 was reported in the group of male alcoholics with self-reported average alcohol consumption of approximately 400g/day for approximately 26 years [65]. Mice that consumed 20% (w/v) ethanol in water for up to 6 months, also showed an increased percentage of activated T cells as measured by increased expression of CD43, Ly6C, rapid IFN-γ response, and increased sensitivity to low levels of TCR stimulation [66, 67]. Taken together, these studies suggest that chronic alcohol-induced T cell lymphopenia increases T cell activation and homeostatic proliferation resulting in increased proportion of memory T cells relative to naïve T cells. In contrast, moderate alcohol increased frequency of lymphocytes (Table 1.1).

Alterations in immunoglobulin (Ig) levels after alcohol consumption have also been observed. An increase in both IgA and IgM levels in heavy drinkers (90-249 drinks/month) compared to light (<9 drinks/month) or moderate drinkers (30-89 drinks/month) was observed in adult males [58]. Similarly, a human study of 460 males and females with 221 alcohol abstainers, 140 light drinkers (1-140g/week), 53 moderate drinkers (141-280g/week), and 46 heavy drinkers (>280g/week) found a dose-dependent increase in serum IgA levels with alcohol consumption [73]. Additionally, spontaneous IgA synthesis from PBMCs isolated from male and female alcoholic patients with liver disease is higher than that from controls [74]. Analogously, in Wistar female rats, acute ethanol administration at 4g/kg intraperitoneally for 30 minutes increased the concentration of IgA in the intestinal lamina propria [75]. Finally, treatment of a mouse hybridoma cell line with 25, 50, 100 and 200mM ethanol for 48 hours resulted in a linear increase in IgM production [76]. The increased production of Ig in alcoholics could be due to the adduction of liver proteins by acetaldehyde and lipid peroxidation of membranes by MDA that result in increased immunogenicity of self-proteins and the potential initiation of auto-immune responses [77]. In contrast to these observations in subjects with alcohol use disorders, IgG, IgM and IgA concentrations, decreased in both men and women following moderate beer consumption [63]. Similarly, lower IgG levels were observed in female and male moderate consumers than in abstainers [73] (Table 1.1).

1.5.3 Impact of alcohol on infection & vaccination

Several lines of evidence suggest that alcohol consumption exerts a dose-dependent impact on the host response to infection. Chronic alcohol abuse leads to increased susceptibility to bacterial and viral infections, most notably a 3- to 7-fold increase in susceptibility [5] and severity [6] of bacterial pneumonia compared with control subjects. Similarly, the incidence of *Mycobacterium tuberculosis* infection among alcoholics is increased [7-9, 11]. Alcohol use has also been shown to drive disease progression in chronic viral infections such as HIV [78] and HCV [10]. In addition, the magnitude of antibody response following vaccination with Hepatitis B is lower in alcoholics compared to controls [79].

This increased susceptibility has been recapitulated in rodent models of chronic alcohol abuse. For instance, increased morbidity and mortality, pulmonary virus titers, and decreased pulmonary influenza-specific CD8 T cell responses were reported in female mice infected with influenza that consumed 20% (w/v) ethanol in their drinking water for 4-8 weeks [80]. Likewise, higher pathogen burden and decreased CD8 T cell immunity was observed in female mice administered ethanol at 15% (w/v) for 5 days and challenged with *Listeria monocytogenes* [81]. Similar results have been seen in SIV infection of male nonhuman primates [81-84]. Significantly lower protein and mRNA levels of macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (CINC), responsible for recruiting immune cells to the lung during early infection as well as delayed neutrophil recruitment were also observed in response to *Streptococcus pneumoniae* or

LPS-induced endotoxemia in male Sprague Dawley rats intraperitoneally injected once with 20% ethanol (5.5g/kg) 30 minutes before infection [85, 86].

In contrast to the studies above, moderate alcohol consumption seems to enhance immune response to infection and vaccination [49, 63, 87-91]. A study of 391 males and females intentionally exposed to five different respiratory viruses showed that moderate alcohol consumption (1-2 drinks/day) was associated with decreased incidence of colds [87]. A second study using a cohort of 4272 male and female individuals found the consumption of wine inversely associated with the risk of common cold [89]. A third retrospective study that followed 899 men reported that non-drinkers were significantly more likely to experience 2 or more episodes of common cold compared with subjects who consumed 1-2.5 alcoholic beverages per day [90]. Moderate beer consumption also enhanced the production of T cell cytokines IL-2, IL-4, IL-10, and IFN-γ, and reduced IFN-γ/IL-10 ratio [63].

In male rats that were administered low acute (0.5g/kg, 5 days) or chronic (0.43g/kg/day, 28 days) doses of ethanol in a liquid diet, results revealed significantly higher T cell responses to *Mycobacterium bovis* and increased clearance of bacterial pathogens compared to controls. In contrast, a high acute (6g/kg for 5 days) or chronic (12g/kg/day for 28 days) dose of ethanol showed an immunosuppressive effect [88]. More recently, moderate daily alcohol consumption for over a year (average BAC 40mg/dL) in a rhesus macaque model of ethanol self-administration enhanced CD4+ and CD8+ T cell as well as IgG responses following Modified Vaccinia Ankara (MVA) vaccination,

whereas chronic alcohol intoxication in this model (average BAC >80mg/dL) suppressed this response [91].

1.5.4 Impact of alcohol on circulating factors

Alcohol consumption modulates plasma levels of several cytokines, chemokines, growth factors, and hormones in a dose-dependent manner. Several large cohort studies (>2000 male and female subjects) have shown a J-shaped association between ethanol consumption and plasma levels of the acute phase protein, C-reactive protein (CRP) [92-94]. Given the association between CRP levels and cardiovascular disease, the decrease in CRP levels observed with moderate alcohol consumption could explain the reduced incidence of cardiovascular disease in individuals who drink in moderation [95]. J-shaped associations of acute phase proteins albumin and transferrin [93] as well as IL-6 with alcohol consumption were also reported [94]. Interestingly, this last study also found a strong inverse linear trend with increasing alcohol consumption in men and women and soluble TNF-α receptors 1 and 2 (sTNF-R1 and sTNF-R2) levels [94]. TNF-receptors, which mediate TNF-α activity, have been associated with increased risk for adverse cardiovascular outcome, therefore cardiovascular benefits of moderate alcohol consumption may be also mediated by a reduction in TNF-α activity (Albert, Glynn et al. 2003).

A multiplex analysis of plasma collected from 24 healthy male subjects after moderate alcohol consumption (50mL of vodka with 200mL orange juice twice daily or only orange juice daily for 4 weeks during dinner) also showed a significant reduction in

acute phase proteins, ferritin and α1-antitrypsin as well as pro-inflammatory cytokines IL-1 receptor antagonist and IL-18 [96]. In contrast, level of anti-inflammatory protein adiponectin increased [96]. Similarly, plasma adiponectin concentration was increased after 28 days of daily consumption of 450mL of red wine compared with de-alcoholized red wine amongst 34 men, in the absence of changes in subcutaneous and abdominal fat contents as well as body weight [97].

In our nonhuman primate model of voluntary ethanol self-administration, chronic ethanol consumption for 32 months by male cynomolgus monkeys with mean daily intakes that reached 4.0g/kg/day (the equivalent of 16 drinks/day and BAC of 400mg/dL) resulted in decreased circulating levels of factors involved in the recruitment of immune cells to the site of infection including chemokines such as CCL3/4, and metalloproteases such as MMP-9 [98]. Decreased IL-2 and CCL5 levels provide insight into possible mechanisms of impaired T cell recruitment and proliferation. Increases in IL-7 and IL-15, which are critical for T cell survival, may be compensatory mechanisms for reduced IL-2 levels. Reduced IgE levels were also observed and may be related to the observed decrease in IgE synthesis regulators, IL-13 and CD40 ligand. Increased levels of CCL11, a potent chemokine for IgE-producing eosinophils, may be compensating the reduced IgE levels [98]. These changes were most apparent at the highest ethanol intakes and BAC.

Using these data, a 3- (adiponectin, α-2-macroglobulin, and complement component 3) and a 14- (CD40, chemokine ligand 5, factor VII, IgE, IGF-1, IL-2, IL-7, CCL3, CCL4, MMP2, kallikrein-related peptidase 3, glutamic-oxaloacetic transaminase 1, thrombopoietin, and VEGF-A) plasma protein biomarker panels that showed 100%

sensitivity and 88% accuracy of ethanol consumption were developed [99]. The same self-administration macaque model also showed that male animals that drank in moderation (1.975g/kg/day) had slightly elevated plasma levels of IL-2, IL-15, IL-12, TNF-α, Regulated on Activation Normal T cell Expressed and Secreted (RANTES), and monokine induced by gamma interferon (MIG) compared to heavy drinkers and controls [91]. These observations could explain why animals drinking moderately generated a more robust response to MVA vaccination compared to controls and animals that drank to intoxication since these factors are critical for lymphocyte proliferation, T cell activation and effector function, and immune cell recruitment.

1.5.5 Mechanisms of dose-dependent modulation of immunity by alcohol

Mechanisms that underlie these changes in circulating factors are starting to emerge. Alcohol-induced alterations in gene expression involved in cytokine signaling pathways were examined in male and female subjects with alcohol dependence (AD; more than 25 drinks/week prior to study; n=10), heavy drinkers (HD; defined as regular alcohol use over the past year of at least 8 drinks/week for women and at least 15 drinks/week for men; n=13), and moderate drinkers (MD; defined as up to 7 drinks/week for women and 14 drinks/week for men; n=17). After normalization, 436 differentially expressed genes in blood samples were identified: 291 genes differed between AD and MD subjects, 240 between AD and HD subjects, but only 6 differed between HD and MD subjects [100]. Many of the differentially expressed genes were involved in the regulation of the immune response by cytokine signaling and the Janus kinase/signal transducers and activators of

transcription (JAK-STAT) pathway. Expression of interferon receptor 2 (*IFNAR2*) was down-regulated in HD subjects compared to MD subjects which could contribute to deficits in both innate and adaptive immunity [100]. IL-15, a cytokine critical for promoting survival, proliferation, and activation of NK and CD8 T cells, was found to be up-regulated in AD subjects but there was no difference between HD and MD subjects [100]. Expression of the receptor for IL-21, which has been reported in autoimmune diseases, was found to be up-regulated in AD subjects [100].

A second study by Joosten *et al.* also analyzed gene expression profiles in PBMCs isolated from 24 healthy male subjects who consumed 50mL of vodka with 200mL orange juice or only orange twice daily for 4 weeks during dinner (considered to be moderate). Pathways involving antigen presentation, B and T cell receptor signaling, and IL-15 signaling were altered with moderate vodka consumption [96]. The most significant change was in glucocorticoid receptor (GR) signaling, which is known to down-regulate immune activity and inflammation by down-regulating NFκB [101]. Indeed, NFκB was down-regulated in the alcohol group compared with the control group [96]. The observed decrease in expression of NFκB is in line with earlier studies examining decreased pro-inflammatory cytokine production with moderate alcohol consumption.

It is also critical to take into consideration that the effects of ethanol on immune function *in vivo* could involve the actions of its primary metabolite, acetaldehyde. Acetaldehyde has been shown to modulate gene expression by binding transcription factors and modifying chromatin structure [102, 103]. Therefore, more studies looking at the effects of ethanol metabolites *in vivo* are needed. Acetaldehyde has also been shown to

affect NFκB-induced cytokine production in various liver cells. In the presence of acetaldehyde, Kupffer cells, the specialized macrophages in the liver, treated with LPS show decreased NFκB activation [104], while hepatic stellate cells, the major producers of collagen that accumulate during hepatic fibrosis, show enhanced NFκB activation [105].

One of the mechanisms by which AUD can interfere with immunity is through micronutrient deficiency. DCs can convert vitamin A to retinoic acid, which is used by T and B cells as a substrate to induce the up-regulation of gut-homing molecules and generation of IgA-secreting B cells [19]. Consequently, deficiency in vitamin A results in the impairment of mucosal responses [19]. In addition, antigen presenting cells convert vitamin D to $1,25(OH)_2VD_3$, a physiologically active form of vitamin D that is highly concentrated in lymphoid tissues [19] where it can modulate function of T and B cells which express vitamin D receptors. Vitamin D deficiency therefore results in reduced differentiation, phagocytosis and oxidative burst, by monocytes as well as defective bactericidal activity by keratinocytes [106, 107].

Vitamin E is one of the most effective antioxidants and its deficiency exacerbates free-radical damage impairing the ability of T cells to respond to pathogenic challenge [108]. Similarly, vitamin C, also an antioxidant, is important for phagocytic activity of neutrophils and monocytes, and enhances T cell responses [109]. Thiamine, also known as vitamin B_1, contributes to the activation of T cells, suppresses oxidative stress-induced NFκB activation in macrophages, and serves as an anti-inflammatory factor [110]. Antigen-specific responses are decreased in folate-deficient humans and animals [111].

Future studies aimed at uncovering the mechanisms underlying dose-dependent modulation of immune function should also investigate changes in gene expression patterns, as well as factors that regulate gene expression including microRNAs and epigenetic changes within specific immune cell populations. For example, levels of miR-155 have been shown to be elevated in macrophages following ethanol exposure and contribute to the high levels of TNF-α [112]. Alcohol-fed rats also show an increase in acetylation of histone 3, which may be associated with increased ROS levels [113]. Additionally, the role of alcohol-induced changes in the microbiome on immunity should be further studied. Finally, an emerging informatics approach that can piece together these extensive data sets and build a network between the immune response elements and the time-course/dose response of ethanol while emphasizing *in vivo* studies from rodent, NHP, and humans is urgently required.

1.6 Impact of alcohol on the gastrointestinal tract

AUD results in gut injury highlighted by increased permeability, nutrient malabsorption, functional alterations in mucosal immune cells, increased risk of colorectal cancer (CRC), and perturbations of the gut microbiome [114]. The intestinal mucosal immune system acts as the first line of defense against enteric pathogens while tolerating food antigens and commensal organisms.

1.6.1 Gastrointestinal mucosal immune system

The small (duodenum, jejunum, and ileum) and large (colon) intestines form a continuous tube that is lined by a single layer of columnar epithelial cells connected together by tight junction proteins. Epithelial cells are responsible for the absorption and digestion of nutrients, secretion of ions and water, and protecting the host from exogenous insults. Undifferentiated cells arising from the crypts continuously renew the surface epithelium. Multipotent stem cells give rise to different types of mature epithelial cells including absorptive enterocytes, mucus-secreting goblet cells, and neuroendocrine cells [115]. Villi, which extend into the lumen and increase the surface area of digestively active epithelium, are present throughout the small intestine, but absent in the large intestine. Throughout the intestine, a mucus layer coats the mucosa to act as a physical barrier and contains mucin glycoproteins that are toxic to microbes. Mucus is thickest in the colon, which harbors by far the largest microbial load and contains two distinct layers: 1) an inner dense layer attached to the epithelial surface where bacteria do not normally penetrate; and 2) an outer loose layer [116]. The frequency and type of leukocyte populations including various T cell subsets and IgA+ plasma cells fluctuate throughout the various segments of the gut resulting in distinct physiological functions and immune responses [115].

There are significant differences between the peripheral and mucosal immune system of the gastrointestinal tract. Due to their anatomical location, mucosal epithelial cells interact closely with lymphoid tissues. The gastrointestinal tract contains distinct compartments of diffuse lymphoid tissue and specially organized structures such as Peyer's patches in the distal portion of the small intestine. Effector mechanisms are also unique in

the following ways: 1) activated memory T cells predominate even in the absence of an infection; 2) presence of multiple effector and regulatory T cell subsets that are distinct from those found in peripheral blood; and 3) production of secretory IgA antibodies. The immunoregulatory environment of the intestines actively dampens immune responses to prevent aberrant inflammatory responses to food and other innocuous antigens. There are also specialized macrophages and DC along the gastrointestinal tract [35]. The gastrointestinal tract also harbors populations of resident commensal microorganisms, referred to as microbiota or microbiome. The gut microbiome protects against infection through competition for resources and by stimulating the development of the immune system and function of the mucosal barrier [117, 118]. A complex interplay between intestinal epithelial cells, immune cells, and microbiome regulates intestinal homeostasis and barrier function.

1.6.2 Alcohol-induced barrier dysfunction

Increased permeability of the gastrointestinal tract as measured by polyethylene glycol clearance was observed after a one-time administration of 20g ethanol in healthy human volunteers [119]. Intestinal hyperpermeability coupled with translocation of luminal contents into the underlying lamina propria causes systemic inflammation impacting several other organs and plays a critical role in the initiation and pathogenesis of ALD [120-122]. Alcohol metabolism by bacteria and intestinal epithelial cells results in acetaldehyde accumulation, which increases phosphorylation of tight junction proteins resulting in their redistribution and functional impairment [123]. Data from *in vitro* studies

using ethanol-treated human intestinal epithelial cell lines indicate that increased permeability is mediated by dysregulated expression and/or distribution of tight junction proteins zonula occludens (ZO)-1 and claudin-1 [124-126], as well as adherens junctions E-cadherin and β-catenin [127, 128]. These *in vitro* findings are in line with clinical and animal model studies that showed reduced expression of claudin-1, occludin, and ZO-1 in the duodenum, ileum, and colon [129, 130], in part due to increased expression of microRNA-212 [125].

Oxidative stress from ethanol metabolism by CYP2E1 results in accumulation of harmful ROS, which in turn leads to an increase in CREB phosphorylation and activation of Snail. *In vitro* studies using intestinal cell lines showed inhibition of Snail prevented alcohol-induced hyperpermeability suggestive of its critical role in barrier function [131]. Increased expression of myosin light chain kinase (MLCK) and phosphorylation of mitogen activated protein kinase (MAPK) proteins also play a role in alcohol-induced increase in intestinal permeability [132].

These observations suggest that immune defects seen in individuals with AUD could also be mediated by nutritional deficiencies in addition to barrier defects and functional changes in immune cells. AUD also affects nutrients absorption by decreasing secretion of digestive enzymes [133]. This is further compounded by the fact that subjects with AUD often derive most of their caloric intake from alcohol limiting their supply of nutrients necessary for structural maintenance of intestinal epithelial cells [134]. However, how these changes contribute to increased susceptibility to infection in individuals with AUD is not completely understood.

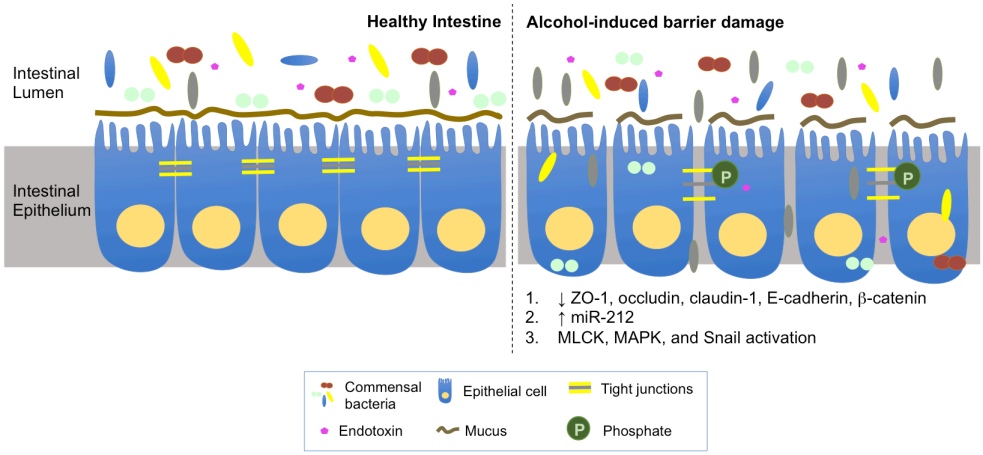


Figure 1.2: Alcohol-induced barrier dysfunction. Summary of the impact of chronic alcohol consumption on the intestinal barrier.

1.6.3 Impact of alcohol on intestinal immunity

The negative impact of alcohol consumption on small and large intestine mucosal immunity has been appreciated for several decades. In the 1970s, ethanol-induced structural alterations in the small intestine and colonic mucosa of heavy drinkers were first described [135, 136]. A significant loss of lymphocytes from gut associated lymphoid tissue and increased susceptibility to intestinal pathogens have been shown in both in animal and clinical studies [137-144]. Specifically, duodenal biopsies obtained from patients with chronic alcohol abuse showed an increase in the number of B-lymphocytes in the lamina propria, as well as a decrease in the number of intraepithelial T-lymphocytes, and macrophages in comparison to biopsies from healthy controls [137]. A more recent study showed a reduction in CD161+ mucosa-associated invariant T cells that was accompanied by hyperactivation (increased expression of CD69) and defective antibacterial cytokine and cytotoxic responses in AUD patients with increased intestinal permeability and liver disease compared to healthy controls [141].

Similar observations were made in rodent models of AUD. Ethanol-fed mice showed a marked loss of lymphocytes from gut-associated lymphoid tissue [138] and increased susceptibility to the intestinal intracellular pathogen *Listeria monocytogenes* [139]. Numbers of CD3+ lymphocytes, IgA+ plasma cells, and macrophages were reduced in the ileal lamina propria of young rats following *in utero* and early post-natal lactational exposure to ethanol [140]. A rat model of acute alcohol and burn injury showed a significant suppression in T cell IL-2 and IFN-γ production, as well as proliferation in gut-associated lymphoid tissue [143]. A study utilizing ethanol-fed mice found, in addition to

a reduction in T-lymphocyte numbers, an increase in apoptosis in the jejunal and colonic lamina propria compared to control animals [144].

Studies using a rhesus macaque model of chronic binge alcohol administration via intra-gastric catheter model found a decrease in absolute numbers of jejunal lamina propria T-lymphocytes, accompanied by increases in T-lymphocyte proliferation and turnover following 3 months of chronic alcohol consumption [142]. In addition, using our macaque model of voluntary ethanol self-administration, we showed that chronic ethanol consumption resulted in a dose-dependent decreased production of IL-2, IL-17, TNFα, and IFNγ by colonic CD4+ and CD8+ T cells potentially due to increased expression of microRNA miR-155 [56].

1.6.4 Association between alcohol consumption and colorectal cancer

AUD is associated with a 70% greater risk for developing colorectal cancer (CRC) [145-147]. Interestingly, moderate alcohol consumption may be associated with a reduced risk of colorectal cancer [148], however more studies are warranted to understand the underlying mechanisms. Metabolism of ethanol by intestinal epithelial cells increases ROS production creating an ideal tumor environment. Dysregulated immune response also provides a microenvironment that promotes tumor growth. Production of proinflammatory cytokines such as IL-6 and IL-18 in addition to further release of ROS by immune cells from increased LPS exposure exacerbates inflammation, creating the ideal pro-tumorigenic environment. Studies using the human colorectal carcinoma HCT116 cell line showed ethanol inactivates GSK3-β, leading to increased nuclear translocation of β-catenin, which

contributes to tumor progression and metastasis via production of MCP-1/CCR2 [149]. Microbial dysbiosis, bacterial overgrowth, intestinal hyperpermeability, and chronic inflammation due to continuous pro-inflammatory cytokine/chemokine production together prime the tumor microenvironment. Finally, genetic polymorphisms make some individuals more susceptible to tumor progression, DNA damage, generation of DNA adducts, and genomic instability [150].

1.6.5 Alcohol and dysbiosis

The human microbiome is composed of bacteria, archaea, viruses, and eukaryotic microbes, which greatly impact the majority of physiologic functions. The intestinal microbiome is dominated by major bacterial phyla: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. A healthy balanced microbiome is important for host defense against invading pathogens by preventing pathogenic microbes from colonizing the intestine through competition for essential nutrients, space and attachment sites on the epithelium, and secretion of toxins to inhibit the growth of other bacteria. Furthermore, these microorganisms are crucial for physiological processes including digestion of food, synthesis of essential nutrients, renewal of gut epithelial cells, as well as the development and regulation of the immune system. Several human diseases have been shown to be significantly associated with alterations in the gut microbiome including rheumatoid arthritis, colorectal cancer, obesity, and diabetes [151].

AUD alters the fecal microbiome in both humans [152-155] and rodent models [121, 156]. Studies have reported increased abundance of Gram-negative anaerobic

bacteria and endospore-forming rods [152], as well as bacteria overgrowth in AUD subjects compared to healthy controls [153]. Small intestine bacterial overgrowth alters motility and delays gastric emptying resulting in increased gastrointestinal symptoms including nausea, constipation, and diarrhea. Lower abundances of *Bacteroidetes* and higher abundances of *Proteobacteria* have been shown in colonic biopsies and fecal samples from patients [154, 155] as well as mice with ALD [121]. Alcohol treatment increased the total number of observed bacteria when compared to control mice, and the largest difference came from increases in *Enterobacteriaceae*, such as *Enterococcus* [122].

Changes in microbial communities are believed to contribute to higher intestinal permeability since antibiotic treatment prevents the ethanol-induced increase in intestinal permeability in rats [157]. Furthermore, germ-free mice do not exhibit alterations in intestinal permeability or liver damage following acute alcohol intake [122]. On the other hand, conventionalization of germ-free mice with intestinal contents from alcohol-treated conventional mice induced inflammation in the small intestine and liver. Interestingly, treatment with a high fiber diet decreased intestinal permeability following alcohol consumption [122]. The contributions of the gut microbiome to the development of barrier dysfunction could be due to the higher activity of bacterial acetaldehyde dehydrogenase relative to that of bacterial alcohol dehydrogenase, which results in the accumulation of acetaldehyde. The major anaerobic bacteria responsible for the production of carcinogenic levels of acetaldehyde from ethanol in the colon and rectum belong to the genera *Ruminococcus*, *Collinsella*, *Prevotella*, *Coriobacterium*, and *Bifidobacterium* [158].

1.7 Thesis overview and aims

The rhesus macaque model of voluntary ethanol self-administration provides a unique opportunity to investigate the impact of chronic ethanol consumption on immunity and intestinal homeostasis. We previously showed moderate consumption resulted in a more robust T-cell and antibody vaccine response to Modified Vaccinia Ankara (MVA), while heavy drinkers generated blunted T-cell and antibody response compared to controls [159]. Since the molecular mechanisms underlying these responses are not completely understood, I first compared the transcriptomes of peripheral blood mononuclear cells (PBMCs) isolated from controls, moderate, and heavy drinkers post-MVA vaccination. Furthermore, the dose- and site-dependent impact of chronic ethanol consumption on intestinal homeostasis is largely understudied. Therefore, in the second half of my dissertation, I characterized the ethanol-induced changes in mucosal gene expression and bacterial composition of intestinal biopsies collected from all major gut sections. Finally, I investigated how chronic ethanol consumption alters the transcriptome of gut-resident lamina propria lymphocytes. Data from these studies provide novel insight into how ethanol leads to mucosal damage and ultimately disrupts intestinal homeostasis, and have the potential to guide efforts to develop new interventions to improve health outcomes in alcoholics.

Chapter 2: Alcohol consumption modulates host defense in rhesus macaques by altering gene expression in circulating leukocytes

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Abstract

Several lines of evidence indicate that chronic alcohol use disorder leads to increased susceptibility to several viral and bacterial infections whereas moderate alcohol consumption decreases incidence of colds and improves immune responses to some pathogens. In line with these observations, we recently showed that heavy ethanol intake (average blood ethanol concentrations (BECs) >80 mg/dl) suppressed, whereas moderate alcohol consumption (BEC <50 mg/dl) enhanced T and B-cell responses to Modified Vaccinia Ankara (MVA) vaccination in a nonhuman primate model of voluntary ethanol consumption. To uncover the molecular basis for impaired immunity with heavy alcohol consumption and enhanced immune response with moderate alcohol consumption, we performed a transcriptome analysis using PBMCs isolated on day 7 post-MVA vaccination, the earliest time point at which we detected differences in T-cell and antibody responses. Overall, chronic heavy alcohol consumption reduced expression of immune genes involved in response to infection and wound healing, and increased expression of genes associated with the development of lung inflammatory disease and cancer. In contrast, chronic moderate alcohol consumption upregulated expression of genes involved in immune response and reduced expression of genes involved in cancer. In order to uncover mechanisms underlying the alterations in PBMC transcriptomes, we profiled the expression of microRNAs within the same samples. Chronic heavy ethanol consumption altered the levels of several microRNAs involved in cancer and immunity and known to regulate expression of mRNAs differentially expressed in our dataset.

Introduction

Alcohol use disorder (AUD) results in a significant increase in both incidence and severity of infections such as bacterial pneumonia, tuberculosis, hepatitis C virus, and HIV [10, 160, 161]. Similarly, chronic ethanol consumption in rodents results in increased pathogen burden and impaired ability to clear *Listeria monocytogenes* [81], *Mycobacterium tuberculosis* [162], and influenza virus [80]. Likewise, rhesus macaques given ethanol via intragastric cannula show increased simian immunodeficiency virus replication compared to controls [163]. Increased vulnerability to infection in individuals with AUD is due to changes in barrier function as well as innate and adaptive immunity [164]. Dysregulation of tight junction proteins in the lungs and gut increases permeability, leading to bacterial translocation into the alveolar space and circulation, respectively [165, 166]. In addition, AUD results in the inhibition of phagocytic functions, reduction of chemotaxis and aberrant cytokine production, and diminished lymphocyte numbers and antigen-specific responses [167].

In contrast, data from several studies support a beneficial role for moderate alcohol consumption on immunity. Moderate alcohol consumption is associated with decreased incidence of the common cold in humans [87, 90, 168] as well as improved bacterial clearance and increased delayed cutaneous hypersensitivity response following infection with *Mycobacteria bovis* in rats [88]. Recently, we showed using a macaque model of ethanol self-administration [33] that moderate consumption resulted in a more robust T-cell and antibody vaccine response to Modified Vaccinia Ankara (MVA), while heavy drinkers generated blunted T-cell and antibody response compared to controls [159].

Moreover, we showed that the dose-dependent effects of ethanol on the immune response to the MVA vaccine were independent of changes in frequency of major immune cell subsets. Specifically, numbers of circulating lymphocyte, monocyte, and neutrophil as well as the frequency of CD4 T cell, CD8 T cell, and CD20 B cells (and their naïve and memory subsets) did not differ between control and ethanol consuming animals [159]. Instead, we detected changes in the expression of several microRNAs (miRNAs) associated with development and function of the immune system, suggesting that ethanol dose-dependent modulation of immunity is mediated by changes in gene expression. Therefore, in this study, we compared the transcriptomes of PBMCs isolated from controls, moderate, and heavy drinkers on day 7 post-MVA vaccination.

Our results revealed that chronic heavy ethanol consumption was associated with significant downregulation of genes involved in immune response to infection and wound healing as well as upregulation of genes associated with development of obstructive lung disease and cancer. In contrast, chronic moderate alcohol consumption was associated with reduced expression of genes involved in neoplasia and the upregulation of genes involved in host defense. In order to uncover mechanisms underlying the alterations in PBMC transcriptomes, we also examined changes in miRNA expression. Our analysis showed that chronic heavy ethanol consumption altered the expression of several miRNAs whose targets were differentially expressed in our data set and are involved in cancer progression and immune function. Overall, data presented in this manuscript provide novel insight into the mechanisms by which excessive alcohol consumption interferes with immune

responses, and exacerbates co-morbidities such as poor wound healing, lung disease, and cancer, while moderate consumption improves immunity.

Materials and methods

Ethics statement

This study was performed in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare and the United States Department of Agricultures. All animal work was approved by the ONPRC Institutional Animal Care and Use Committee (IACUC).

Animal studies and sample description

The animal model and vaccination strategy were previously described [159]. Briefly, we used schedule-induced polydipsia to establish reliable self-administration of 4% (w/v) ethanol in 8 male rhesus macaques [33]. Four animals served as controls for a total of 12 animals. Following a 4-month induction period, animals were allowed a choice of 4% ethanol or water for 22hr/day every day for 12 months. In this nonhuman primate model of voluntary self-administration, animals segregate naturally into heavy and moderate drinkers within 2-3 months, and these patterns remain stable for at least 12 months [169]. In this specific cohort, ethanol-drinking animals segregated into two cohorts, n=4 each based on average blood ethanol (BEC) values: moderate drinkers with average BEC of 22.3-48.8 mg/dl and heavy drinkers with average BEC of 90-126 mg/dl [159]. All 12 animals were vaccinated with MVA prior to induction of ethanol and again after 7 months of open access to ethanol. We used PBMCs isolated 7 days after booster vaccination for

RNA and microRNA expression analysis. Only 3 animals from each group had sufficient numbers of PBMCs for RNA sequencing.

RNA isolation and mRNA library preparation

Total RNA was isolated from PBMCs using the miRNeasy kit (Qiagen, Valencia, CA). One microgram of RNA was used to generate libraries using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA). Poly(A)-enriched mRNA was fragmented followed by cDNA synthesis with random hexamers. This product underwent end-repair, adapter ligation, and size selection using AMPure XP beads (Beckman Coulter Inc., Brea, CA) to isolate cDNA templates of 320 nucleotides that were amplified by PCR. Each library was prepared with unique index primers for multiplexing and subjected to single-end 100 bp sequencing on the HiSeq2500 platform (Illumina, San Diego, CA).

Small RNA library preparation

One microgram of total RNA extracted as described above underwent adapter ligation and primer hybridization prior to cDNA synthesis and PCR amplification using the NEBNext Small RNA Library Prep Set for Illumina Kit (New England Biolabs, Ipswich, MA). Size selection was performed with AMPure XP beads (Beckman Coulter Inc., Brea, CA) to isolate cDNA templates of 140 nucleotides. Each library was prepared with unique index primers for multiplexing and subjected to single-end 50 bp sequencing on the HiSeq2500

platform (Illumina, San Diego, CA). We were unsuccessful in generating one library from one of the heavy drinkers.

RNA-Seq analysis

Data analysis was performed with the RNA-Seq workflow module of the *systemPiperR* package available on Bioconductor [170, 171]. Quality reports were generated with the *seeFastq* function. RNA-Seq reads were mapped with the splice junction aware short read alignment suite Bowtie2/Tophat2 [172, 173] against the *Macaca mulatta* genome sequence from Ensembl [174]. For the alignments, we used default parameters of Tophat2 optimized for mammalian genomes. Raw expression values in form of gene-level read counts were generated with the *summarizeOverlaps* function [175]. Here, we counted only reads overlapping exonic regions of genes, discarding reads mapping to ambiguous regions of exons from overlapping genes. Given the non-stranded nature of RNA-Seq libraries, the read counting was performed in a non-strand-specific manner. The RNA sequencing data have been deposited in NCBI's Sequence Read Archive (SRA) under the accession number SRP064253 (http://www.ncbi.nlm.nih.gov/sra). Analysis of differentially expressed genes (DEGs) was performed with the GLM method from the *edgeR* package [176, 177]. DEGs were defined as those with a fold change of ≥ 2 and a false discovery rate (FDR) of ≤ 0.05. Enrichment analysis of functional annotations was performed to identify significant biological pathways including gene ontology (GO) terms and disease biomarkers using MetaCore™ software (GeneGo, Philadelphia, PA).

Small RNA-Seq analysis

Adaptor contaminations were removed (trimmed) from the reads using the *preprocessReads* function from the *systemPipeR* package. The preprocessed reads were aligned with Bowtie2 [172, 173] against the *Macaca mulatta* genome sequence with settings optimized for miRNA alignments, including tolerance of multiple mappings. Reads overlapping with miRNA gene ranges were counted with the *summarizeOverlaps* function as described above, but in a strand-specific manner. The miRNA gene coordinates, required for this step, were downloaded from miRBase (Release version 19). The small RNA sequencing data have been deposited in NCBI's SRA under the accession number SRP064540 (http://www.ncbi.nlm.nih.gov/sra). Differentially expressed miRNA genes were identified with *edgeR* as described above. TargetScan was used to predict genes for each differentially expressed microRNA with a high context ratio of 0.95. These targets were then compared with our list of differentially expressed genes among the three groups of rhesus macaques. These combinations of differentially expressed mRNA and miRNA were then segregated based on the directions of fold changes.

Gene validation via qRT-PCR

cDNA was synthesized from RNA isolated as above, using High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). mRNA expression was determined by quantitative reverse transcription PCR using Taqman primer and probe kits specific for *Macaca mulatta* cDNA sequences and a StepOnePlus instrument (Life Technologies, Grand Island, NY). mRNA expression levels of LYZ (Rh02902590),

PTGS2 (Rh02787804), THBS1 (Rh00962902), KLF4 (Rh02847953), TLR4 (Rh01060206), CD14 (Rh03648680), CD163 (Hs00174705), and FN1 (Rh02621780) for each sample were calculated relative to control RPL32 mRNA expression using ΔCt calculations.

Results

Heavy alcohol consumption leads to significant changes in gene expression

There were 514 DEGs between controls and heavy drinkers (C7-H7), 479 of which were annotated with 356 downregulated and 123 upregulated genes with heavy drinking. We identified 368 DEGs between moderate and heavy drinkers (M7-H7), 347 of which were annotated with 290 downregulated and 57 upregulated genes with heavy drinking. Finally, of the 60 DEGs between controls and moderate drinkers (C7-M7), 47 were annotated with 29 downregulated and 18 upregulated with moderate ethanol consumption (Fig. 2.1A).

Heavy ethanol consumption was associated with the largest changes in gene expression compared to both controls and moderate consumption (Fig. 2.1A, 2.1B), with 171 downregulated (Fig. 2.1C), and 26 upregulated DEGs compared to controls and moderate drinkers (Fig. 2.1D). The overwhelming majority of the downregulated (Fig. 2.1E) and upregulated (Fig. 2.1F) DEGs showed a 2-4 fold-change in expression. To confirm the RNA-Seq results, 8 genes differentially expressed with chronic heavy ethanol consumption (*LYZ*, *PTGS2*, *THBS1*, *KLF4*, *TLR4*, *CD14*, *CD163*, and *FN1*) were selected for confirmation using qRT-PCR. Changes in expression level of all 8 DEGs were confirmed. In order to better understand the biological relevance of these gene expression changes, we conducted functional enrichment analysis using the MetaCore™ pathway-mapping tool.

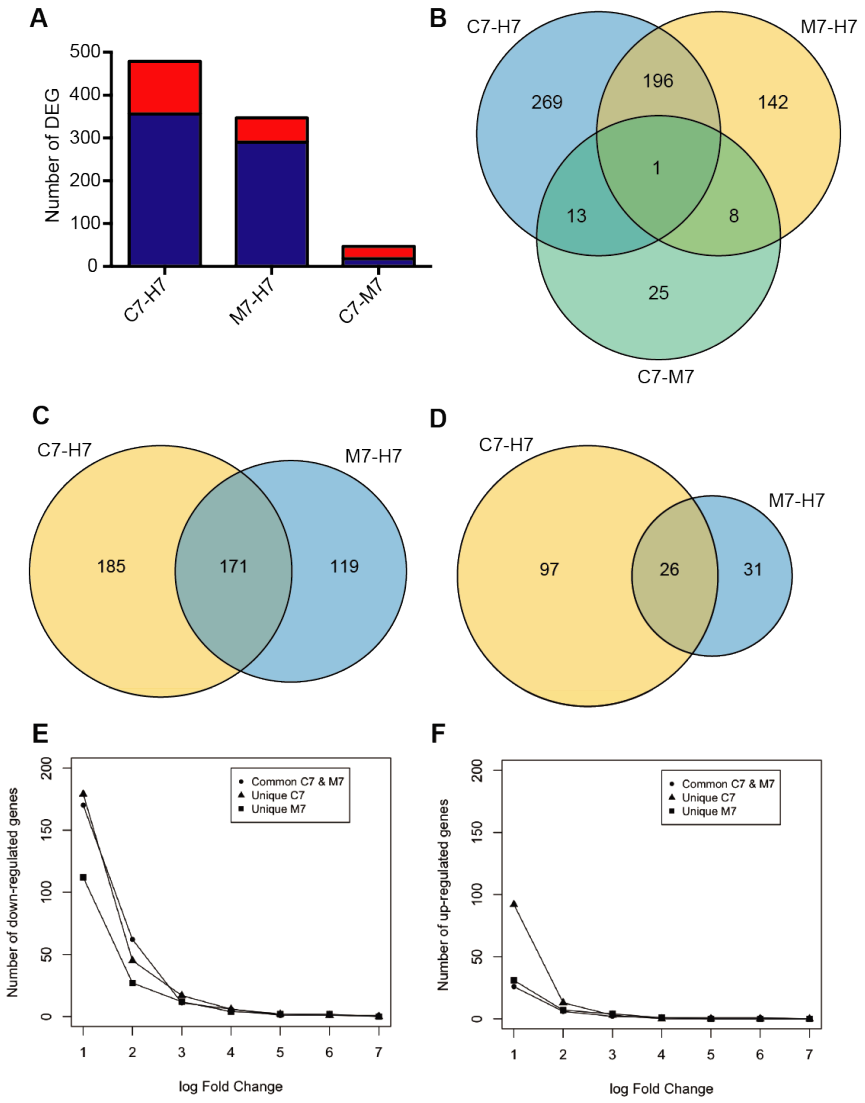


Figure 2.1: Peripheral blood mononuclear cell (PBMC) gene expression. (A) Bar graph showing the number of downregulated (blue) and upregulated (red) differentially expressed genes (DEGs) for each comparison. (B) Venn diagram depicting the overlap of the annotated DEGs between controls (C), moderate drinkers (M), and heavy drinkers (H) on day 7 after MVA vaccination (C7, M7, and H7 respectively). (C) Venn diagram depicting the overlap between genes that are downregulated with heavy alcohol consumption compared to controls and moderate drinkers. (D) Venn diagram depicting the overlap between genes that are upregulated with heavy alcohol consumption compared to controls and moderate drinkers. (E, F) Fold change of (E) downregulated and (F) upregulated genes with heavy drinking.

Heavy alcohol consumption downregulates genes that promote host defense compared to both moderate drinkers and controls

Of the 171 genes repressed in H7 compared to C7 and M7, 114 mapped to the following GO terms: response to stress, response to wounding, inflammatory response, response to lipid, and positive regulation of response to external stimulus (Fig. 2.2A). Several DEGs mapping to "response to stress" encode microbial sensors, notably formyl peptide receptor 2 (*FPR2*, FC=128.2), *TLR4* (FC=5.6), *CD14* (FC=3.8), pyrin domain-containing-3 (*NLRP3*, FC=3.3), *TLR8* (FC=2.9), and nucleotide-binding oligomerization domain-containing-2 (*NOD2*, FC=2.4). Other DEGs encode immune receptors such as cadherin EGF LAG seven-pass G-type receptor-1 (*CELSR1*, FC=30.6), syndecan-2 (*SDC2*, FC=7.8), plasminogen activator receptor (*PLAUR*, FC=6.5), macrophage scavenger receptor-1 (*MSR1*, FC=5.7), IL-1 receptor type-1 (*IL1R1*, FC=5.7), IL-13 receptor alpha-1 (*IL13RA1*, FC=4.8), *CCR1* (FC=4.5), and neuropilin-1 (*NRP1*, FC=3.4). Additional DEGs encode chemokines, cytokines, growth factors, and antimicrobial peptides, including matrix metalloproteinase-1 (*MMP1*, FC=8.6), *CXCL8* (FC=7.5), oncostatin-M (*OSM*, FC=4.4), *IL1B* (FC=3.7), vascular endothelial growth factor-A (*VEGFA*, FC=5.2), heparin-binding EGF-like growth factor (*HBEGF*, FC=3.4), and S100 calcium binding protein-8/9 (*S100A8/9*, FC =3.6/2.9).

Several genes listed above also mapped to "response to wounding" and "inflammatory process" and play a role in wound healing (Fig. 2.2B). For instance, *CELSR1*, *SDC2*, *VEGFA*, *HBEGF*, and *NRP1* promote wound closure [178, 179], while *NOD2*, *NLRP3*, *CD14*, coagulation factor plasminogen activator inhibitor-2 (*SERPINB2*,

FC=17.6), complement component 5a receptor-1 (*C5AR1*, FC=4.0) and 3a receptor-1 (*C3AR1*, FC=2.9), and *IL1B* (which activates *CXCL8*, FC=7.5) promote chemotaxis and leukocyte extravasation into injury sites [180-183].

Additional analysis showed that 89 genes map to these disease categories: obstructive lung diseases, pathologic processes, hypersensitivity, bacterial infection and mycoses, and inflammation (Fig. 2.2C). Of the 52 genes mapping to "obstructive lung diseases" (Fig. 2.2D), 24 interact with each other (Fig. 2.2E), and are important for lung homeostasis, notably *MMP1* (FC=8.6, involved in lung alveolar epithelial cell migration [184]), *VEGFA* (important for alveolar structure [185]), cathelicidin antimicrobial peptide (*CAMP*/*LL37*, FC=2.8), and aryl hydrocarbon receptor (*AHR*, FC=2.7, regulates apoptosis of lung epithelial cells [186]). DEGs mapping to "pathological processes" include Annexin-2 receptor (*ANXA2R*, FC=17.1), cysteine-rich secretory protein LCCL domain-containing-2 (*CRISPLD2*, FC=9.7), epiregulin (*EREG*, FC=9.2), triggering receptor expressed on myeloid cells-1 (*TREM1*, FC=4.7), and *HBEGF*, which are involved in protection against cancer, sepsis, endotoxin shock, and necrotizing enterocolitis [187-191].

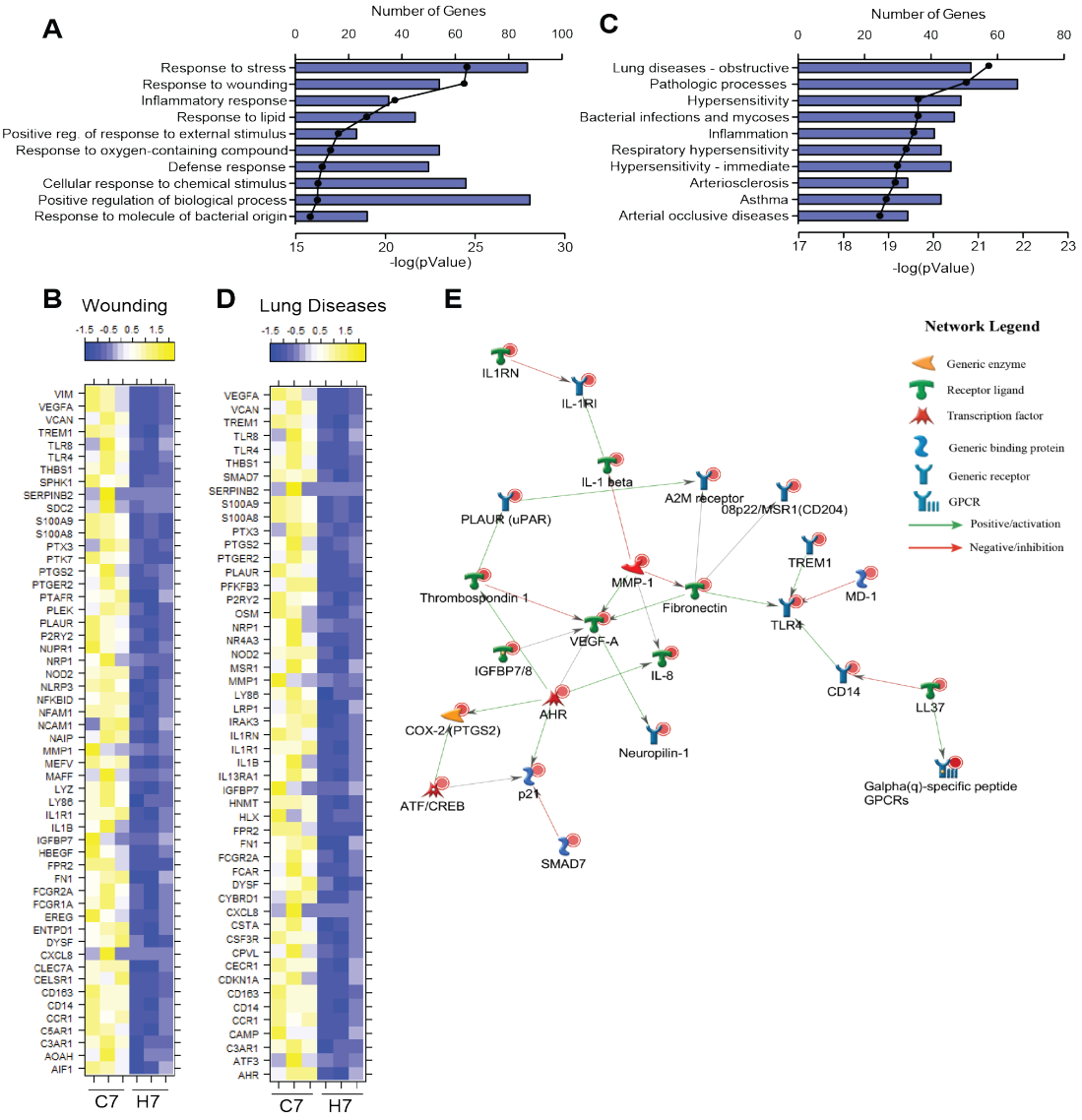


Figure 2.2: Chronic heavy alcohol consumption downregulates genes that promote wound healing and contribute to obstructive lung diseases compared to controls and moderate drinkers. (A) Bar graph displaying the 10 most significant GO terms associated with the 170 genes downregulated with heavy ethanol consumption (H7) compared to controls and moderate drinkers (C7 and M7). Line represents the -log(p value) associated with each GO term. (B) Heatmap of DEGs between H7 and C7 in the "response to wounding" GO term. (C) The 10 most significant diseases by biomarkers associated with the 170 genes downregulated with heavy ethanol consumption (H7) compared to controls and moderate drinkers (C7 and M7). (D) Heatmap of DEGs between H7 and C7 that mapped to "lung diseases-obstructive" category. (E) Network of DEGs that mapped to "obstructive lung diseases" and show direct interactions.

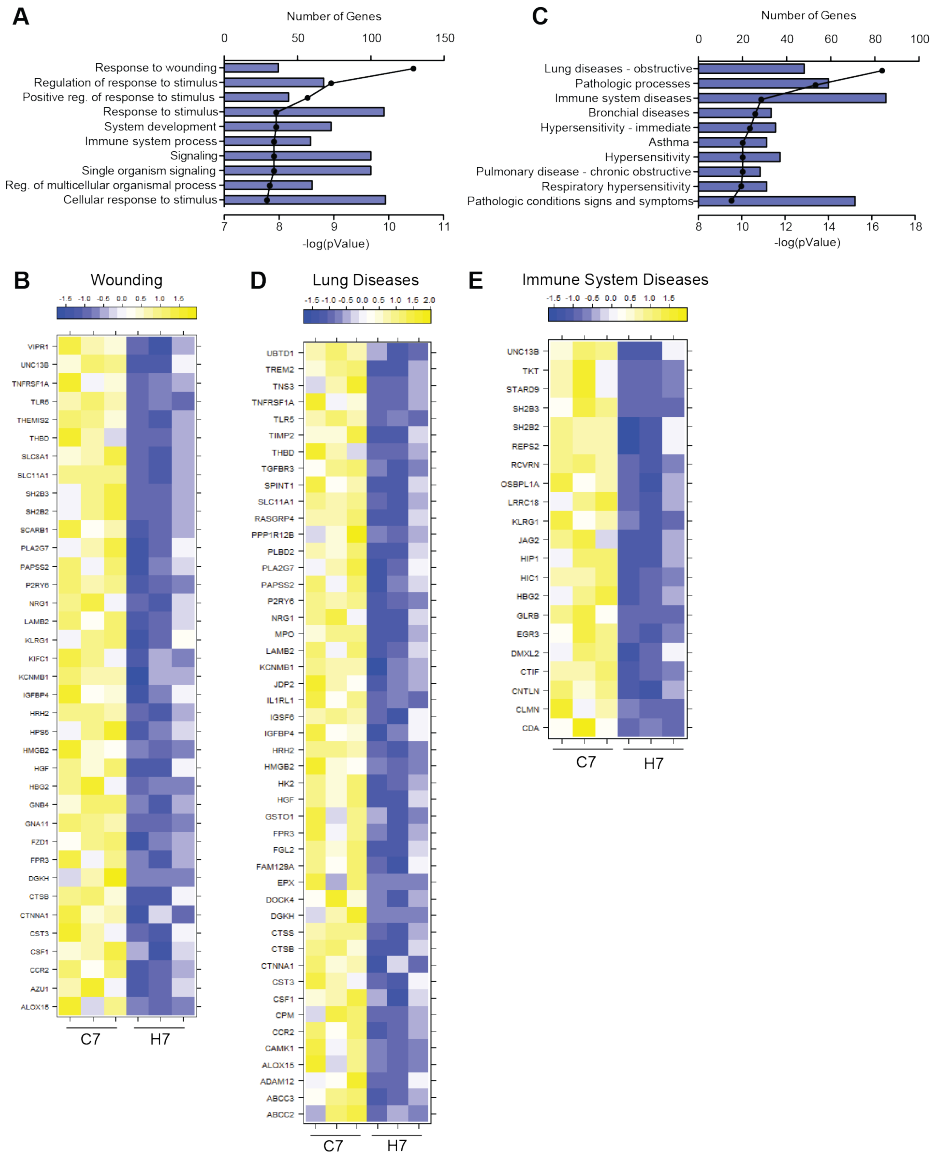
Heavy drinking downregulates genes that promote wound healing and protect against chronic disease compared only to controls

Of the 185 DEGs repressed in H7 compared to C7, 128 mapped to these GO terms: response to wounding, regulation of response to stimulus, positive regulation of response to stimulus, response to stimulus, and system development (Fig. 2.3A). The 37 genes mapping to "response to wounding" play an important role in wound healing (Fig. 2.3B) including diacylglycerol kinase (*DGKH*, FC=5.8, regulates fibroblast migration [192]), catenin alpha-1 (*CTNNA1*, FC=3.4, promotes wound repair in bronchial epithelial cells [193]), metallopeptidase inhibitor-2 (*TIMP2*, FC=3.4, involved in wound closure [194]), solute carrier family-11 (*SLC11A1*, FC=2.2, regulates macrophage activation in cutaneous wounds [195]), *CSF1* (FC=2.1, involved in neoangiogenesis [196]), and hepatocyte growth factor (*HGF*, FC=2.0, accelerates wound re-epithelialization [179]). One highly downregulated gene mapping to "response to stimulus" is eosinophil peroxidase (*EPX*, FC=22.6), a potent toxin for bacteria and parasites [197].

Further analysis showed 106 genes mapped to these disease categories: obstructive lung diseases, pathological processes, immune system diseases, bronchial diseases, and immediate hypersensitivity (Fig. 2.3C). Genes in "obstructive lung diseases" play an important role in lung function (Fig. 2.3D). For instance, IL-1 receptor-like-1 (*IL1RL1*, FC=9.2), *TLR5* (FC=6.0), arachidonate 15-lipoxygenase (*ALOX15*, FC=5.5), *TREM2* (FC=2.6), and TNF-receptor superfamily-1A (*TNFRSF1A*, FC=2.0) promote host defense against bacterial infection in the lungs and regulate lung inflammation, whereas *HGF* (FC=2.0) promotes lung regeneration after injury [179]. Polymorphisms in

myeloperoxidase (*MPO*, FC=7.4) and A Disintegrin and Metalloproteinase domain-12 (*ADAM12*, FC=4.5) modulate the development of lung cancer [198, 199].

Several DEGs that mapped to "immune system diseases" also mapped to "obstructive lung diseases" and "pathological processes" including C-type lectin domain family-10A (*CLEC10A*, FC=4.8) and ATP-binding cassette subfamily-C-2/3 (*ABCC2*/*3*, FC=2.9/3.2), which play a role in antigen recognition and presentation [200, 201]; as well as EGF-like module receptor-1 (*EMRI*, FC=3.7) and interleukin-12 receptor-beta-2 (*IL12RB2*, FC=2.8), which are critical for host defense [202]. Genes unique to this category (Fig. 2.3E) regulate inflammation such as leucine-rich repeat-containing-18 (*LRRC18*, FC=104.5) and hemoglobin subunit gamma-2 (*HBG2*, FC=8.9 [203, 204]); as well as lymphocyte proliferation and differentiation including SH2B adaptor-3 (*SH2B3*, FC=2.0 [205]) and killer cell lectin-like receptor subfamily-G1 (*KLRG1*, FC=2.4 [206]).



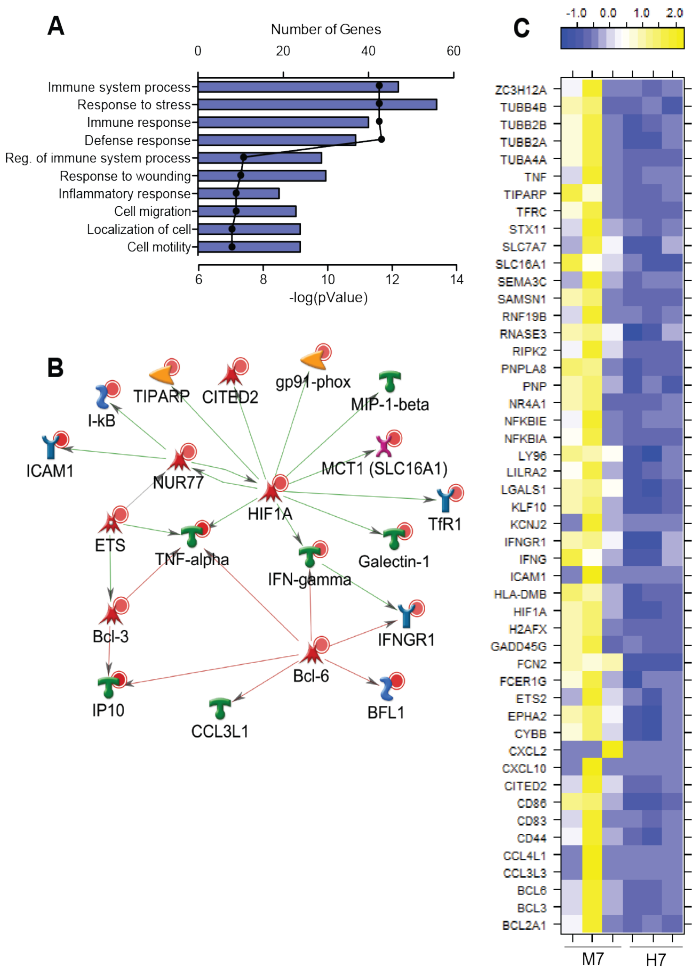
<u>Figure 2.3</u>: Alcohol abuse uniquely downregulates additional genes that promote wound healing and contribute to obstructive lung diseases. (A) Bar graph displaying the 10 most significant GO terms to which the 186 DEGs downregulated with heavy ethanol consumption (H7) compared to controls only (C7). Line represents the -log(p value) for each GO term. (B) Heatmap of DEGs between H7 and C7 mapping to the GO term "response to wounding". (C) Bar graph displaying the 10 most significant Diseases by Biomarker to which the 186 DEGs downregulated with heavy drinking (H7) compared to controls (C7) only. Line represents the -log (p value) for each disease category. (D) Heatmap of the DEGs between H7 and C7 mapping to the "lung diseases-obstructive" disease category. (E) Heat map of the 21 DEGs between H7 and C7 mapping to "immune system diseases".

Heavy alcohol consumption reduces expression of genes that regulate the immune system compared to moderate consumption only

Of the 119 genes downregulated in H7 compared to M7, 66 mapped to the following GO terms: immune system processes, response to stress, immune response, defense response, and regulation of immune system processes (Fig. 2.4A). In total, 47 mapped to immune system-related GO terms, 20 of which interact with each other (Fig. 2.4B). DEGs mapping to these GO terms are involved in: 1) lymphocyte activation and recruitment (*CXCL10*, FC=17.3; *SLC16A1*, FC=3.2 [207]; sterile alpha-motif-domain Src homology-domain nuclear localization signals-1, *SAMSN1*, FC=3.7 [208]; *ICAM1*, FC=5.5; *CD83*, FC=3.0 [209]); 2) antimicrobial response (*TNFA*, FC=7.9; ficolin-2, *FCN2*, FC=6.5 [210]; *MD2*, FC=2.8); and 3) regulation of gene expression (v-ets avian erythroblastosis virus oncogene homolog-2, *ETS2*, FC=2.5; B-cell lymphoma 2-related protein-A1, *BCL2A1*, FC=5.4 [211]; B-cell CLL/lymphoma-3/6, *BCL3*/*6*, FC=2.5/2.0 [212, 213]; Kruppel-like factor-10, *KLF10*, FC=3.0 [214]; nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha, *NFKBIA*, FC=2.9 [215]).

Several genes mapping to "immune system process" (Fig. 2.4C) also map to "response to stress", notably hypoxia-inducible factor-1-alpha (*HIF1A*, FC=3.0) that regulates expression of genes that counter oxidative stress and *ETS2*, which is induced by shear stress to preserve integrity of microvascular walls [216]. Additional notable DEGs that only mapped to "response to stress" included Gadd45-gamma (*GADD45G*, FC=8.4, important in anti-tumor immune responses [217]) and BMX non-receptor tyrosine kinase

(*BMX*, FC=11.5, promotes tight junction formation in epithelial cells during chronic hypoxia [218]).



<u>Figure 2.4</u>: Heavy alcohol consumption downregulates genes associated with regulation of the immune system. (A) Bar graph displaying the top 10 significant GO terms associated with the 122 genes downregulated with heavy ethanol consumption (H7) compared to moderate drinkers (M7) only. Line represents the -log(p value) of each GO term. (B) Network image of DEGs that mapped to the GO term "immune system processes" and directly interact with one another. (C) Heatmap of the DEGs between H7 and M7 that mapped to the "immune system processes" GO term.

Heavy drinking increases expression of genes associated with impaired wound healing, cardiovascular disease and cancer

Within the 26 genes upregulated in H7 compared to both C7 and M7 (Fig. 2.5A), several encode transcription factors associated with skin, colorectal, breast, and lymphoma cancers, notably IFN alpha-inducible protein 27-like-1 (*IFI27L1*, FC=68.0 [219]), *AXIN2* (FC=2.2 [220]), lymphoid enhancer-binding factor-1 (*LEF1*, FC=2.0 [221]), Meis homeobox-1 (*MEIS1*, FC=2.4 [222]), and four-and-a-half LIM domains-1 (*FHL1*, FC=2.1 [223]). Interestingly, increased expression of retinoid X receptor-gamma (*RXRG*, FC=5.2), which is associated with sensation seeking [224], a behavioral trait common among alcoholics, was detected. Another overexpressed gene was serum deprivation protein response (*SDPR*, FC=2.8), which induces deformation of plasma membrane invaginations impairing endocytosis and potentially antigen presentation [225]. Finally, expression of regulator of G-protein signaling-18 (*RGS18*, FC=3.3), growth factor-independent 1B transcription repressor (*GFI1B*, FC=2.3), and rho guanine nucleotide exchange factor-4 (*ARHGEF4*, FC=6.1), which play a role in megakaryocyte differentiation [226, 227], were also increased.

Of the 97 genes upregulated in H7 compared to only C7, 26 mapped to the following GO terms: response to wounding, regulation of body fluids, platelet activation, wound healing, and platelet degranulation. Genes in "response to wounding" (Fig. 2.5B) include connexin-43 (*GJA1*, FC=11.0), a gap junction associated with impaired wound healing [228]; *IL17F* (FC=6.7), which can delay wound closure [229]; and P-selectin (*SELP*, FC=2.3), a glycoprotein highly expressed in wounds [230]. Genes with roles in

cardiovascular disease mapped to the disease category "infarction" including carbonic anhydrase III (*CA3*, FC=5.2), glycoprotein VI (*GP6*, FC=2.9), phosphodiesterase-6H (*PDE6H*, FC=2.6), and integrin-alpha-2b (*ITGA2B*, FC=2.6 [231-234]). Furthermore, heavy ethanol consumption upregulated genes associated with cancer, notably transient receptor potential cation channel-M1 (*TRPM1*, FC=14.9), tripartite motif containing-31 (*TRIM31*, FC=9.8), *RGS6* (FC=7.6), and *CLDN5* (FC=2.2 [235-237]).

Twenty-one of the 31 genes upregulated in H7 compared to only M7 mapped to "neuro-ectodermal tumors" (Fig. 2.5C). These genes are either expressed at high levels in cancer including delta-like-1 (*DLK1*, FC=7.3 [238]) and insulin receptor substrate-1 (*IRS1*, FC=4.8 [239]); or involved in progression of cancer such as phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor-2 (*PREX2*, FC=11.7 [240]), latent transforming growth factor-beta-binding protein-2/3 (*LTBP2*/*3*, FC=3.6/2.6 [241, 242]), and stromal antigen-3 (*STAG3*, FC=2.3 [243]).

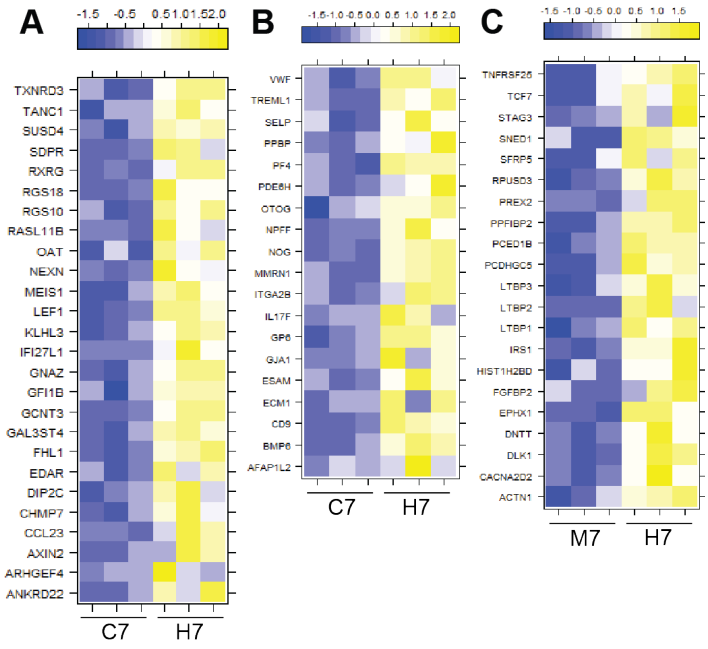


Figure 2.5: Alcohol abuse upregulates genes that interfere with wound healing and contribute to cancer. Heatmaps of the DEGs upregulated with heavy ethanol consumption (H7) compared to: (A) both controls (C7) and moderate (M7) drinkers; (B) controls (C7) only and mapped to the GO term "response to wounding"; and (C) moderate (M7) drinkers only and mapped to the GO term "neuro-ectodermal tumors".

Moderate drinking activates genes associated with immunity and represses genes associated with cancer compared to controls

Of the 29 annotated genes upregulated in M7 compared to C7 (Fig. 2.6A), four play a role in chemotaxis: *CXCL3* (FC=50.3, critical for leukocyte chemotaxis), *IL1A* (FC=23.4, recruitment of neutrophils), *CCL3* (FC=17.1, recruits T-cells [244]), and *CCL4L1*/*2* (FC=8.1/5.5, trafficking of NK cells [245]). Other genes significantly upregulated in M7 include acute phase protein pentraxin-3 (*PTX3*, FC=15.3 [246]); ceruloplasmin (*CP*, FC=3.7) and granzyme A (*GZMA*, FC=2.2), expressed primarily by NK cells and play a role in host defense [247, 248]; and *GJA1* (FC=9.6), important for barrier function [249].

Several of the 18 genes downregulated in M7 compared to C7 (Fig. 2.6B) are involved in cancer progression including transmembrane protein-98 (*TMEM98*, FC=286.9), serine-protease temperature requirement-A1 (*HTRA1*, FC=24.1), DNA nucleotidylexotransferase (*DNTT*, FC=5.7), *MMP9* (FC=3.5), and ten-eleven translocation-1 (*TET1*, FC=3.0 [250-254]). Interestingly, *ABCA9* (FC=6.9), retinoic acid-binding receptor-related orphan receptor-C (*RORC*, FC=3.6), 2'-5'-oligoadenylate synthetase-2 (*OAS2*, FC=2.6), *CD84* (FC=2.2), and myxovirus resistance protein-1 (*MX1*, FC=2.2), which play a role in innate immunity, were downregulated in M7.

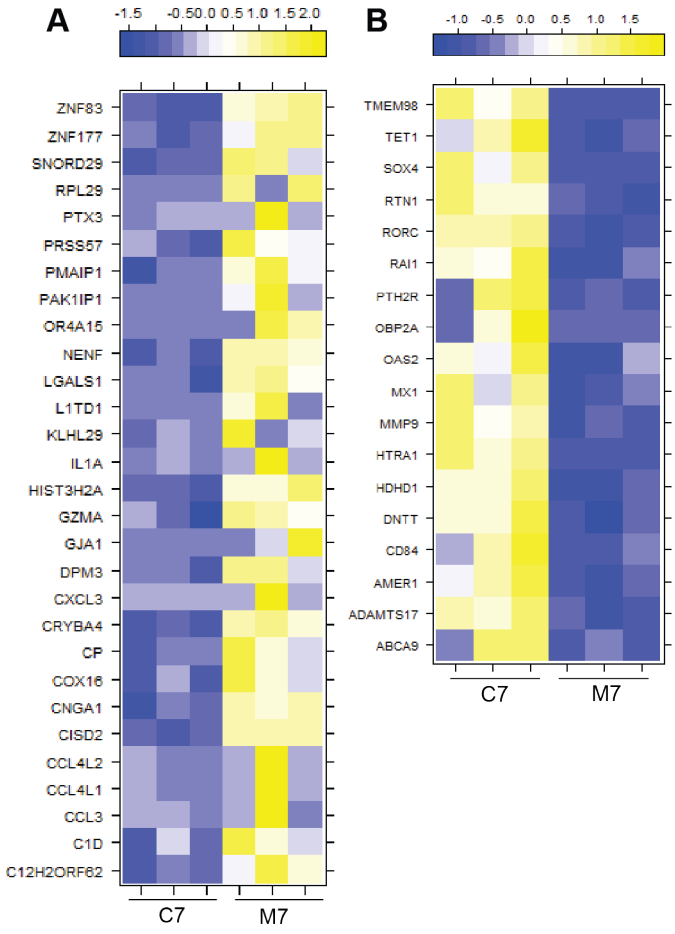


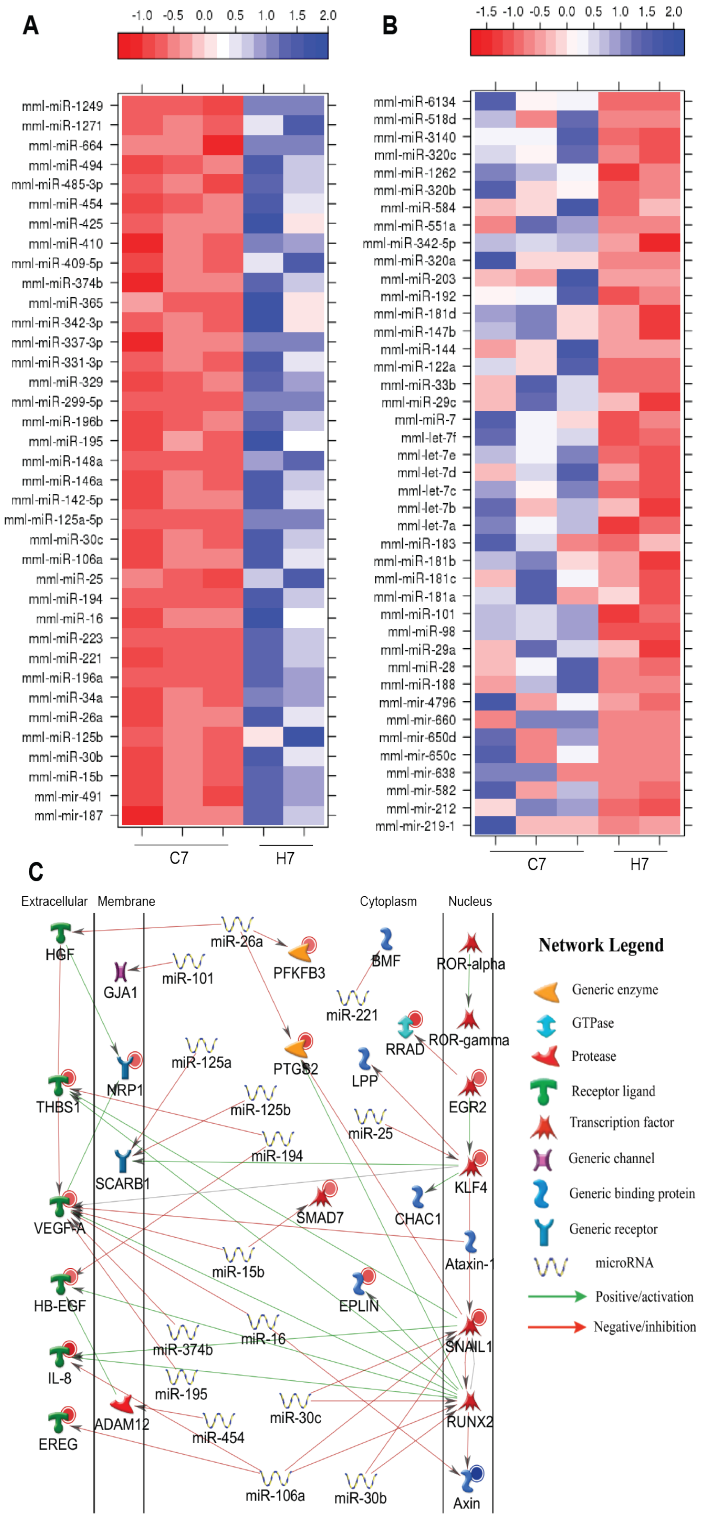
Figure 2.6: Moderate ethanol consumption modulates genes associated with immune response. (A) Heatmap of the DEGs uniquely activated with moderate drinking (M7) compared to controls (C7). (B) Heatmap of the DEGs uniquely repressed with moderate drinking (M7) compared to controls (C7).

Heavy ethanol consumption alters the expression of microRNAs involved in cancer and immune function.

To begin uncovering the mechanisms underlying changes in gene expression regulation with moderate and heavy ethanol consumption, we compared the miRNA expression profiles of the same PBMCs isolated from controls, moderate, and heavy drinkers on day 7 post-MVA vaccination. MiRNAs are ~22 nucleotide long endogenous RNAs that target mRNAs for translational repression or degradation [255], and several reports indicate that ethanol can modulate miRNA expression [256]. As described for mRNA expression, the largest differences in miRNA expression were observed between controls and heavy drinkers, and only a few miRNAs were differentially expressed between controls and moderate drinkers. Interestingly, no differentially expressed miRNAs were detected between heavy and moderate drinkers. There were 79 differentially expressed miRNAs between controls and heavy drinkers, 37 of which were upregulated (Fig. 2.7A) and 42 were downregulated (Fig. 2.7B).

Importantly, 53 of these miRNAs have mRNA targets within our dataset. Heavy drinking led to the upregulation of 29 microRNAs known to regulate 25 target mRNAs that were downregulated in our dataset. A subset of these mRNA-miRNA pairs is shown in Fig. 2.7C; for a complete list, please refer to Table 2.1. Some of the downregulated mRNAs were targeted by several differentially expressed miRNAs. For instance, miR-16, miR-15b, miR-195, and miR-374b target *VEGFA*, which was downregulated >5-fold. Similarly, miR-30b and miR-30c target *SH2B3*, downregulated 2-fold, while miR-125a and miR-

125b both target *SCARB1*, which was downregulated >3-fold. Of the 24 microRNAs downregulated with heavy drinking, 7 were associated with an increase in their mRNA targets (Fig. 2.7C and Table 2.1). For example, the downregulated miR-101 targets *GJA1*, which was upregulated 11-fold with heavy drinking. MiR-144 targets *AXIN2*, which was upregulated 2-fold. MiR-183 and miR-202 both target *ROBO2*, which was upregulated more than 3-fold. Finally, miR-29a, miR-29b, and miR-29c all target *SH3PXD2A*, which was upregulated 4-fold in our dataset.


A
-1.0 -0.5 0.0 0.5 1.0 1.5 2.0
mml-miR-1249
mml-miR-1271
mml-miR-664
mml-miR-494
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mml-miR-454
mml-miR-425
mml-miR-410
mml-miR-409-5p
mml-miR-374b
mml-miR-365
mml-miR-342-3p
mml-miR-337-3p
mml-miR-331-3p
mml-miR-329
mml-miR-299-5p
mml-miR-196b
mml-miR-195
mml-miR-148a
mml-miR-146a
mml-miR-142-5p
mml-miR-125a-5p
mml-miR-30c
mml-miR-106a
mml-miR-25
mml-miR-194
mml-miR-16
mml-miR-223
mml-miR-221
mml-miR-196a
mml-miR-34a
mml-miR-26a
mml-miR-125b
mml-miR-30b
mml-miR-15b
mml-mir-491
mml-mir-187
C7
H7
B
-1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0
mml-miR-6134
mml-miR-518d
mml-miR-3140
mml-miR-320c
mml-miR-1262
mml-miR-320b
mml-miR-584
mml-miR-551a
mml-miR-342-5p
mml-miR-320a
mml-miR-203
mml-miR-192
mml-miR-181d
mml-miR-147b
mml-miR-144
mml-miR-122a
mml-miR-33b
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mml-miR-7
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mml-let-7e
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mml-let-7c
mml-let-7b
mml-let-7a
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mml-miR-181b
mml-miR-181c
mml-miR-181a
mml-miR-101
mml-miR-98
mml-miR-29a
mml-miR-28
mml-miR-188
mml-mir-4796
mml-mir-660
mml-mir-650d
mml-mir-650c
mml-mir-638
mml-mir-582
mml-mir-212
mml-mir-219-1
C7
H7
C
Extracellular
Membrane
Cytoplasm
Nucleus
HGF
GJA1
miR-26a
miR-101
PFKFB3
BMF
ROR-alpha
miR-221
RRAD
ROR-gamma
miR-125a
PTGS2
LPP
NRP1
THBS1
miR-125b
EGR2
miR-25
miR-194
SCARB1
KLF4
SMAD7
CHAC1
VEGF-A
Ataxin-1
miR-15b
HB-EGF
EPLIN
miR-374b
miR-16
SNAIL1
IL-8
miR-195
miR-30c
RUNX2
ADAM12
miR-454
EREG
miR-106a
miR-30b
Axin
Network Legend
Generic enzyme
GTPase
Protease
Receptor ligand
Transcription factor
Generic channel
Generic binding protein
Generic receptor
microRNA
Positive/activation
Negative/inhibition

(Previous page) Figure 2.7: Heavy ethanol consumption changes expression of several microRNA. Heatmaps of the (A) upregulated and (B) downregulated microRNAs with heavy drinking (H7) compared to controls (C7). (C) Network image of a subset of the differentially expressed microRNAs and their mRNA targets that were both differentially expressed in our study.

Differentially Expressed MicroRNAs	Differentially Expressed mRNA Targets
Down-regulated	Up-regulated
miR-144	*AXIN2*
miR-203	*AFAP1L2, ROBO2*
miR-183	*ROBO2*
miR-29c	*SH3PXD2A*
miR-29a	*SH3PXD2A*
miR-101	*GJA1*
miR-29b	*SH3PXD2A*
Up-regulated	Down-regulated
miR-26a	*HGF, PTGS2, NTN4, RP2, RAB3IP, CHAC1, PFKB3, DAPK1*
miR-25	*GRHL1, KLF4, FAM20C*
miR-142-5p	*GAS7, BVES, ATP13A3, PRRG4, RTN1, LPP, PRDM8, NRG1*
miR-374b	*GAS7, VEGFA, ATXN1, DUSP6, DUSP8*
miR-125b	*SCARB1, CCR2, FAM129B, C19orf39*
miR-410	*RORA, SMAD7, RAPGEF2, SASH1, SNAI1, PTX3*
miR-485-3p	*BAIAP2*
miR-16	*SMAD7, TNFSF13B, VEGFA, ATP13A3, RASGEF1B*
miR-425	*FSCN1*
miR-30c	*SH2B3, SNAI1, RUNX2, HLX, EAF1, RRAD*
miR-106a	*LIMA1, NTN4, OSM, WFS1, EGR2, EREG, DOCK4, FAM129A, RORC, IL8*
miR-342-3p	*ZAK, NEURL1B*
miR-494	*IRAK3, ZFHX3*
miR-125a-5p	*SCARB1, CCR2, FAM129B, C19orf38*
miR-148a	*NRP1, B4GALT5, SESTD1*
miR-15b	*SMAD7, TNFSF13B, VEGFA, ATP13A3, RASGEF1B, AATK*
miR-221	*BMF, NRG1*
miR-365	*KCNQ1*
miR-454	*ZAK, ATXN1, RTN1, ADAM12, ACSL1, WDFY3, MB21D2, TMEM170B*
miR-195	*SMAD7, TNFSF13B, VEGFA, ATP13A3, RASGEF1B, AATK*
miR-329	*ATXN1*
miR-34a	*CLEC10A, REPS2*
miR-30b	*SH2B3, SNAI1, RUNX2, HLX, EAF1, RRAD*
miR-194	*HBEGF, THBS1, ZFHX3*
miR-223	*OLFM1, SLC8A1*

Table 2.1: mRNA-miRNA pairs. Table shows the downregulated miRNAs that target upregulated mRNAs as well as the upregulated miRNAs that target downregulated mRNAs in our datasets.

Discussion

Using a macaque model of ethanol self-administration, we recently showed that heavy ethanol consumption suppresses, whereas moderate ethanol consumption enhances, T and B-cell responses to MVA [33]. The goal of this study was to uncover the molecular mechanisms underlying this dose-dependent effect. We used RNA-Seq to identify changes in gene expression on day 7 following vaccination with MVA, the earliest time point at which we detected differences in antibody and T-cell responses [33]. We also sequenced small RNA molecules to gain insight into mechanisms underlying the changes in gene regulation. Overall, our study revealed robust changes in gene and miRNA expression between control, moderate, and heavy drinkers, with fewer changes between moderate drinkers and controls compared to either group versus heavy drinking status.

The changes in gene expression reported herein provide novel insight into the reduced immune response to vaccination and the increased vulnerability to infection seen in humans with AUD. Specifically, we detected large decreases in the expression of microbial sensors that are critical in the detection of bacterial peptides (*FPR2/3* [257]), LPS (*MD2*, *CD14*, *TLR4*), bacterial flagellin (*TLR5*), and certain helminths and filoviruses (*CLEC10A* [200]). There was also decreased expression of genes important for antigen presentation (*ABCC2/3*, *CLEC10A* [200, 201]); recruitment of immune cells (*C3AR1*, *C5AR1*, *CCR2*, *CXCL3*, *CXCL8*, *CXCL10*, *CCL3*, *CCL4L1/2*, *ICAM1* [182, 244, 258-260]); and soluble mediators that play a role in response to infection (*TNFA*, *TNFRSF1A*, *IFNG*, *IFNGR1*, *IL1R*, *IL1R1*, *IL1RL1*, *IL1B*, *S100A8/9*, *LL37*, *EPX*, *GRZMA* [197, 261]). As previously described [100], expression of interferon signaling interferon receptor-2

(*IFNAR2*) was downregulated in heavy drinkers, which would contribute to deficits in both innate and adaptive immunity.

We also found decreased expression of lymphocyte activation markers including *CD83* and *KLRG1* [206]. In our previous study, excessive ethanol consumption suppressed whereas moderate ethanol consumption enhanced MVA-specific IgG responses [159]. These defects in antibody production could be partially explained by the decreased expression of transcription factors *BCL3* and *BCL6*, which are important in germinal center formation, isotype class switching, and hypermutation [212, 213], *SH2B3*, which regulates B-cell development [205], and *SAMSN1*, an adapter protein involved in immune cell signaling [208]. Reduced expression of *KLF10*, which suppresses regulatory T-cells by upregulating TGFβ [214], could further explain the reduction in T and B-cell responses.

Our study also revealed insight into the mechanisms by which moderate alcohol consumption stimulates immunity. Animals that drank moderate amounts of ethanol showed increased expression of chemokines *CCL3* and *CCL4L1*, which signal through CCR5 to recruit memory T-cells [244], and *IL1A* and *CXCL3*, which recruit neutrophils. Although a vigorous innate immune response is critical, it is equally important for the host to minimize damaging inflammation. *PTX3*, which plays a role in the resolution of inflammation [246], and the NFκB inhibitor, *NFKBIA*, were significantly upregulated with moderate drinking. These observations are in line with previous studies that showed that moderate alcohol consumption in humans significantly alters genes involved in B-cell, T-cell, and IL-15 signaling pathways, and attenuates NFκB signaling pathways in leukocytes [262].

Several of the genes that were differentially expressed with ethanol consumption have previously been described as being important for mounting immune responses to vaccination. For instance, studies that investigated yellow fever vaccine (YF-17D)-induced signatures in blood of healthy adults have reported significant increases in the expression of proinflammatory mediators *CXCL10* and *IL1A* and complement gene *C3AR1*, which were found to be predictive of robust vaccine responses [263]. Another study identified *ETS2* as an additional key regulator of the early innate immune response to YF-17D [264]. In our study, we observed a 17-fold downregulation of *CXCL10*, a 3-fold downregulation of *C3AR1*, and a 2.5-fold downregulation of *ETS2* with excessive ethanol consumption, which could explain the suppression of vaccine responses in this cohort. In contrast, we detected a 23-fold upregulation of *IL1A* in moderate drinkers compared to controls, re-enforcing the association of this marker with successful immune responses.

Our gene expression analysis is also in line with clinical observations linking alcohol abuse with impaired wound healing [265], increased susceptibility of wound infections [266], and delay of wound closure [267]. This defect has significant clinical ramifications since half the emergency room trauma cases involve alcohol exposure [268]. Previous studies showed that ethanol exposure at the time of traumatic injury impairs wound closure via decreased pro-inflammatory cytokine release, neutrophil recruitment, and phagocytic function [265]. Our gene expression data support and extend these earlier observations. We detected significantly decreased expression of multiple components of the innate immune system that play a critical role in the prevention of wound infections including pattern recognition receptors (*FPR2/3*, *NALP3*, *NOD2*, *MD2*, *CD14*, *TLR4*,

TLR5, *TLR8*, *CLEC10A*, *SCARB1*), proinflammatory cytokines and their receptors (*TNFRSF1A*, *IFNG*, *IFNG1*, *IL1R*, *IL1R1*, *IL1RL1*, *IL1B*, *IL13RA1*), as well as chemokines and their receptors (*CXCL2*, *CXCL8*, *CXCL10*, *CCR1*, *CCR2*, *OSM*, *CSF3R*, *CSF1*).

Earlier studies suggested the disruption of VEGF signaling and reduced expression of HIF-1α in endothelial cells with chronic alcohol consumption interferes with wound closure [267]. Our gene expression data also show a significant decrease in the expression of both of those genes with excessive ethanol consumption. Moreover, we detected fewer transcripts of *NRP1*, which is expressed by endothelial cells and associates with the VEGF receptor to promote angiogenesis and wound repair [269]. We also report decreased expression of *CELSR1* and *SDC2* that have been shown to promote effective wound repair [178, 270] as well as secreted proteins such as fibronectin and MMP-1 which promote platelet aggregation, angiogenesis, and tissue remodeling [271, 272]. The expression of additional growth factors, *HGF* and *HBEGF*, which promote angiogenesis and tissue regeneration, was also significantly decreased [179]. Furthermore, heavy alcohol abuse increased expression of additional genes known to interfere with wound healing (*PSEL*, *VWF*, *CX43*, *IL17* [229, 230, 273]).

Additionally, our data is in line with clinical observations that heavy alcohol consumption is associated with increased incidence of chronic obstructive pulmonary disease (COPD [274, 275]), lung injury in response to inflammatory insults [276], acute respiratory distress syndrome (ARDS [277]), and risk of mortality in acute lung injury patients [278]. Impaired immunity and increased oxidative stress, both consequences of AUD, are considered risk factors for COPD and ARDS [279]. Our transcriptome analysis

provides new insight into the mechanisms that underlie increased susceptibility and severity of lung injury and chronic lung inflammatory diseases. Heavy ethanol consumption was associated with downregulation of several genes important for maintaining lung homeostasis that can be categorized into: transcription factors (*AHR*, *P21*, *RORG*, *ATF/CREB*); receptors (*TREM1*, *FCGR*, *NOR1*); immune signaling molecules (*IL1R*, *IL1B*, *CXCL8*, *CD14*, *CD163*, *GCSF*, *TNFR1*, *TLR5*, *ALOX15*, *ADAM12*); transporters (*SLC11A1/16A1*); and growth factors (*VEGF*, *HBEGF*, *HGF*). *AHR* and *RORG* play a role in suppressing lung inflammation [186, 280] while decreased *P21* expression is associated with hypoxia-induced lung disease [281]. In addition, transcripts associated with immune genes that promote host defense against pulmonary infection (*ALOX15*, *CD14*, *GCSF*, *TREM1*, *TLR5*, *NRAMP1*, *TNFR1*) were reduced with heavy drinking [282-285]. Critical growth factors important for repairing lung injury were also downregulated. Decreased levels of VEGF correlate with loss of alveolar structure in emphysema patients [185] and a compromised integrity of the alveolar-capillary barrier [286]. Finally, HGF is important for lung development and also promotes regeneration after lung injury in animals [179].

Chronic alcohol consumption is associated with an increased risk of cardiovascular disease [287] and stroke [288]. Our analysis has revealed increased expression of genes implicated in heart disease including *CLDN5*, *VWF*, *PDE6H*, *ITGA2B*, and *CA3* [231, 233, 234, 289, 290], as well as megakaryocyte differentiation (*RGS18*, *GFI1B*, and *ARHGEF4* [226, 227]). *ITGA2B* is used as a biomarker for myocardial infarction risk and therapeutic modulation of phosphodiesterases is one strategy for treating cardiovascular disease.

Interestingly, claudin-5 levels are reduced in human and mice models of cardiomyopathy; therefore, its increased expression here might be a compensatory mechanism.

Finally, heavy alcohol use is a major risk factor for liver, head and neck, and colorectal cancers [13-15]. Our gene expression analysis showed increased expression of several genes that promote cancer progression with chronic heavy alcohol consumption (*LTBP2/3*, *IRS1*, *SFRP5*, *LEF1*, *DLK1*, *STAG3*, and *H2B*). Higher levels of *IRS1* are found in hepatocellular carcinoma, breast, ovarian, and colorectal cancers [291-294]. Increased expression of *LEF1* is associated with human endometrial tumors, prostate, and colon cancer [295-297]. *DLK1* is also expressed at higher levels in colon adenocarcinomas, pancreatic islet carcinomas, and small cell lung carcinomas [238]. In contrast, moderate alcohol consumption is associated with a reduced risk of developing kidney cancer [298], Hodgkin's lymphoma [299], and thyroid cancer [300]. Our study revealed moderate drinking repressed genes associated with reduced cancer incidence including *TMEM98* [253], *HTRA1* [252], *DNTT* [250], *TET1* [251], and *MMP9* [254].

We also investigated differences in miRNA expression levels between the three experimental groups. MiRNAs can modulate gene expression through translational repression or degradation of target mRNAs and play a critical role in regulating immune function [301]. We identified several differentially expressed miRNAs with validated mRNA targets present in our RNA-Seq dataset. Interestingly, and as described for mRNA, several of these upregulated miRNAs have also been implicated in the development and progression of cancer. For instance, *miR-494* has been shown to be upregulated in hepatocellular carcinomas and promote proliferation in tumor cells [302], while *miR-106a*

is upregulated in gastric, colorectal, and pancreatic cancers in humans [303]. As described previously in human hepatocytes and cholangiocytes treated with ethanol [304], *miR-34a* was upregulated greater than 5-fold in our dataset. Furthermore, ethanol-induced hypomethylation of the *miR-34a* promoter, which results in increased expression of this miRNA, plays a role in the development of alcoholic liver disease [304].

Moreover, several upregulated miRNAs in our dataset are involved in modulating immune responses. For example, and as we recently reported [159], *miR-221* was upregulated in PBMCs of heavy drinkers. We previously showed that increased levels of *miR-221* resulted in decreased expression of transcription factors STAT3 and ARNT, which in turn regulate expression of VEGF, G-CSF, and HGF [91, 159]. Indeed, *VEGFA* and *HGF* were downregulated in this study by 5- and 2-fold, respectively. Another target of *miR-221*, *RGS6*, was also downregulated in this study. In addition, upregulation of *miR-125b* interferes with the innate immune response following LPS stimulation or microbial infection [305]. Finally, *miR-223*, up-regulated 19-fold in our dataset, inhibits NFκB activation, angiogenesis, and endothelial cell proliferation, thereby impairing wound healing [306] and inflammation [307].

Many of the downregulated miRNAs are also involved in cancer. For instance, *miR-203* has been identified as a tumor suppressor and inhibits proliferation in colorectal cancer cell lines [308]. Similarly, expression of *miR-144* is significantly decreased in human lung cancer and inhibits proliferation in lung cancer cell lines [309]. Finally, chronic ethanol feeding in a mouse model of alcoholic steatohepatitis has also led to downregulation of *miR-183* [310]. Additionally, most of the downregulated miRNAs modulate immunity.

MiR-183 levels have been shown to be positively associated with phagocytosis by macrophages [311]. As we reported previously [56, 159], *miR-29a* expression was modulated by heavy drinking. Specifically, *miR29a* was downregulated and its target *SH3PXD2A* was upregulated. Finally, in addition to its role as a tumor suppressor, increased expression of *miR-203* has also been shown to be important for anti-inflammatory responses [312].

In summary, our studies revealed that heavy ethanol consumption results in the downregulation of genes that promote resolution of infection, wound healing, and protect against obstructive lung diseases and cancer whereas, moderate drinkers showed increased expression of genes associated with enhancing immune responses. Heavy drinking status also resulted in upregulation of genes involved in impaired wound healing and cancer progression compared to controls and moderate drinkers, whereas moderate ethanol consumption lowered expression of genes associated with cancer. Moreover, heavy ethanol consumption altered the expression of several miRNAs whose targets were differentially expressed in our data set and are involved in cancer progression and immune function. One of the strengths of this study is that we used an outbred animal model of voluntary self-administration that faithfully recapitulates human behavior and physiology. However, a caveat of the current study is that only 3 animals per group were analyzed. Future studies are needed to extend these observations using a larger cohort of animals to other infectious agents such as influenza. Future studies will also investigate the mechanisms underlying dose-dependent changes in gene expression by uncovering factors regulating gene expression such as epigenetic changes within specific immune cells that an influence

expression of both mRNA and miRNA. Using the nonhuman primate model of alcohol self-administration, longitudinal gene regulation, and epigenetic changes in immune cells and target organs, offer the promise for understanding the complicated and dose-dependent impact of alcohol on immunity and health.

Chapter 3: Concurrent gut transcriptome and microbiota profiling following chronic ethanol consumption in nonhuman primates

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Abstract

Alcohol use disorder (AUD) results in increased intestinal permeability, nutrient malabsorption, and increased risk of colorectal cancer (CRC). Our understanding of the mechanisms underlying these morbidities remains limited because studies to date have relied almost exclusively on short-term heavy/binge drinking rodent models and colonic biopsies/fecal samples collected from AUD subjects with alcoholic liver disease (ALD). Consequently, the dose- and site-dependent impact of chronic alcohol consumption in the absence of overt liver disease remains poorly understood. In this study, we addressed this knowledge gap using a nonhuman primate model of voluntary ethanol self-administration where rhesus macaques consume varying amounts of 4% ethanol in water for 12 months. Specifically, we performed RNA-Seq and 16S rRNA gene sequencing on duodenum, jejunum, ileum, and colon biopsies collected from 4 controls and 8 ethanol-consuming male macaques. Our analysis revealed that chronic ethanol consumption leads to changes in the expression of genes involved in protein trafficking, metabolism, inflammation, and CRC development. Additionally, we observed differences in the relative abundance of putatively beneficial bacteria as well as those associated with inflammation and CRC. Given that the animals studied in this manuscript did not exhibit signs of ALD or CRC, our data suggest that alterations in gene expression and bacterial communities precede clinical disease and could serve as biomarkers as well as facilitate future studies aimed at developing interventions to restore gut homeostasis.

Introduction

The gastrointestinal (GI) tract is the largest mucosal surface composed of intestinal epithelial cells (IEC), immune cells, and microorganisms that together form the most critical barrier between the host and its environment [117, 118]. Interactions between these three components are vital to maintaining selective permeability and immune homeostasis in the gut [313, 314]. Each region of the GI tract is unique in its physiological function and microbial load [115]: the duodenum and jejunum are primarily responsible for absorption and digestion of dietary components; the ileum, the longest segment of the small intestine, harbors the majority of gut-associated lymphoid tissue (GALT); and the colon functions to reabsorb water, eliminate waste, and contains the highest bacterial content.

Alcohol use disorder (AUD) results in gut injury highlighted by increased permeability, perturbations of the gut microbiome, nutrient malabsorption, increased risk of colorectal cancer (CRC), and functional alterations in mucosal immune cells [114]. Data from *in vitro* studies indicate that increased permeability is mediated by dysregulated expression and/or distribution of tight junction proteins, notably, zonula occludens (ZO)-1 and claudin-1 in ethanol or acetaldehyde-treated IEC lines [124-126]. These *in vitro* findings are in line with clinical and animal model studies that showed reduced expression of claudin-1, occludin, and ZO-1 in the duodenum, ileum, and colon [129, 130], in part due to increased expression of microRNA-212, [125]. Increased intestinal permeability coupled with translocation of luminal contents plays a critical role in the initiation and pathogenesis of alcoholic liver disease (ALD) [120-122].

AUD alters the fecal microbiome in both humans [152-155] and rodent models [121, 156]. Early studies reported increased abundance of Gram-negative anaerobic bacteria and endospore-forming rods [152], as well as bacteria overgrowth in AUD subjects compared to healthy controls [153]. Lower abundances of *Bacteroidetes* and higher abundances of *Proteobacteria* have been shown in colonic biopsies and fecal samples from patients [154, 155] as well as mice with ALD [121]. Changes in microbial communities are believed to contribute to higher intestinal permeability since antibiotic treatment prevents the ethanol-induced increase in intestinal permeability in rats [157].

Additionally, AUD affects nutrients absorption by decreasing secretion of digestive enzymes [133]. This is further compounded by the fact that subjects with AUD often derive most of their caloric intake from alcohol limiting their supply of nutrients necessary for structural maintenance of IEC [134]. Consequently, subjects with AUD are often deficient in essential vitamins and minerals that play a crucial role in the modulation of gut mucosal immunity [315]. Furthermore, AUD is associated with a 70% greater risk for developing colorectal cancer (CRC) [145-147]. Finally, studies showed reduced cytokine production by lamina propria lymphocytes with ethanol consumption, suggestive of reduced immune surveillance [56, 143].

Despite these findings, several gaps in our understanding of the impact and mechanisms of ethanol-mediated GI injury persist. For instance, very few studies have examined the impact of AUD on GI tract segments other than the colon. Additionally, the impact of non-heavy alcohol consumption is largely understudied despite the fact it is widespread [20]. Finally, mechanisms of reduced nutrient absorption and the increased risk

for CRC remain unclear. Some of the major obstacles to addressing these questions are: 1) limited access to intestinal biopsies along the entire GI tract from human subjects; 2) ethical consideration in obtaining biopsies along the GI tract from subjects who are non-heavy consumers and don't exhibit disease; and 3) the short duration of ethanol consumption coupled with high ethanol concentration used in rodent studies [316].

To address these questions, we used a nonhuman primate model of voluntary ethanol self-administration where following a 3-month ethanol induction period, animals are given "open-access" to both 4% ethanol and water for 22 h/day for up to 24 months [33, 169]. Because animals voluntarily consume varying doses of ethanol, they naturally segregate into non-heavy and heavy drinkers with intakes that closely mirror human data [169]. After 18 months of chronic heavy ethanol consumption, animals develop ALD as evidenced by elevated liver enzymes and liver inflammation [317]. Using this animal model, we recently reported robust gene expression changes and disruptions in innate immune pathways in peripheral blood mononuclear cells isolated from both male [318] and female [319] heavy drinkers. These data provide potential mechanisms to explain the higher incidence of infection, delay in wound healing, and the increased risk of cardiovascular disease, lung inflammatory disease, and cancer seen in subjects with AUD. We also previously showed that despite the lack of differences in the frequency of intestinal lymphocytes, chronic ethanol consumption resulted in a dose-dependent decreased production of IL-2, IL-17, TNFα, and IFNγ by gut-resident CD4+ and CD8+ T cells [56].

In this study, we sought to uncover dose- and site-dependent ethanol-induced alterations in mucosal gene expression and bacterial composition of intestinal biopsies

collected from all major gut sections from 8 ethanol-consuming and 4 control male rhesus macaques. Our RNA-Seq analysis revealed several gene expression changes associated with protein localization, metabolism, inflammation, and CRC in ethanol-consuming animals. We also found that chronic ethanol consumption led to a dose-dependent decrease in putatively beneficial bacteria and an increase in bacteria associated with inflammation and CRC along the GI tract. Additional *in silico* analyses suggest putative changes in bacterial metabolic pathways. Since none of our animals exhibited clinical signs of CRC or ALD, these data suggest that ethanol-mediated changes in gene expression and bacterial communities could serve as early biomarkers of GI disease.

Materials and Methods

Ethics approval

Tissues were acquired from the Monkey Alcohol Tissue Research Resource (MATRR/matrr.com). All animal work was performed in strict accordance with the recommendations detailed in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare and the United States Department of Agricultures and approved by the Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee (IACUC).

Rhesus macaque model of voluntary ethanol self-administration

We leveraged the Monkey Alcohol Tissue Research Resource (MATRR; http://www.matrr.com/) to conduct the experiments described in this manuscript. Samples from 12 male rhesus macaques 4-5 years of age were used in these studies. Eight of these male rhesus macaques chronically consumed 4% ethanol in water for 12 months and segregated into two cohorts, n=4 each, based on average daily ethanol intake values: non-heavy drinkers with an average daily blood ethanol concentration (BEC) of 22.3-48.8 mg/dl (or mean daily intake of 1.8-2.3 g/kg) and heavy drinkers with an average daily BEC of 90-126 mg/dl (or mean daily intake of 2.8-3.3 g/kg). Classification of the animals into non-heavy and heavy drinkers was previously described in [34, 169] and the Monkey Alcohol Tissue Research Resource (www.matrr.com). Four males served as controls and consumed a calorically-matched maltose dextrose solution. Although controls and ethanol-consuming animals consumed comparable numbers of total calories (Fig. 3.1A), heavy

drinkers consumed a much higher number of ethanol-derived calories compared to non-heavy drinkers (Fig. 3.1B). Mean daily BEC and g/kg consumed (calculated over the entire 12-month open access period) significantly correlated with each other (Fig. 3.1C). All 12 animals were euthanized after 12 months of open access and biopsies were collected from duodenum, jejunum, ileum, and traverse colon, snap frozen, and stored at -80°C until analysis. Biopsies were cut in half: one half was used for RNA isolation and the other half was used for DNA isolation. Plasma samples were also collected at necropsy following 12 months of ethanol self-administration, however fecal samples and luminal contents were not available. Ethanol-consuming animals did not show overt signs of liver damage as assessed by measuring alanine transaminase (ALT) and aspartate transaminase (AST) enzyme levels and histological assessment of liver sections (Fig. 3.1D to F).

RNA sequencing (RNA-Seq)

Total RNA was isolated from duodenal, jejunal, ileal, and colonic biopsies using the Qiagen miRNeasy kit (Qiagen, Catalog #217004). Ribosomal RNA (rRNA) was depleted using the Epicentre Ribo-Zero rRNA Removal kit (Illumina, Catalog #MRZH11124). Libraries were constructed using the Bioo Scientific NEXTflex Rapid Directional RNA-seq kit (Bioo Scientific, Catalog #NOVA-5138-10). Briefly, rRNA-depleted RNA was fragmented and converted to double stranded cDNA. Adapters were ligated and ~300 base pair fragments were amplified by PCR and selected by size exclusion. Each library was labeled with a unique barcode for multiplexing. To ensure proper sizing, quantitation, and quality prior to sequencing, libraries were analyzed on the Agilent 2100 Bioanalyzer.

Multiplexed libraries were subjected to single-end 100 base pair sequencing using the Illumina HiSeq2500 platform.

RNA-Seq bioinformatic analysis

Data analysis was performed with the RNA-Seq workflow module of the *systemPiperR* package available on Bioconductor [170, 171]. Quality reports were generated with *seeFastq*. Reads were mapped with the splice-aware aligner suite Bowtie2/Tophat2 [172, 173] against the *Macaca mulatta* genome from Ensembl [174]. Default parameters of Tophat2 optimized for mammalian genomes were used for alignment (allowing 2% nucleotide mismatches). Raw expression values in form of gene-level read counts were generated with *summarizeOverlaps* [175]. Only reads overlapping exonic regions of genes were counted, discarding reads mapping to ambiguous regions of exons from overlapping genes.

Analysis of differentially expressed genes (DEG) was performed with the generalized linear model likelihood ratio test method from the *edgeR* package [176, 177]. To determine the impact of ethanol consumption on gene expression, we compared the transcriptome of controls versus that of ethanol-consuming animals. DEG were defined as those with a fold change of ≥ 2 and a Benjamini-Hochberg-controlled false discovery rate (FDR) of <0.05. To confirm the RNA-Seq results, expression levels of 8 DEG that play a role in ALD, CRC, and host defense/inflammation in the: 1) ileum [*CD2* (Rh02839718_m1), *CCL19* (Rh02621767_m1), *CCR6* (Rh02788181_s1), *CCR7* (Rh03985963_s1), and *BCL6* (Rh02839507_m1)]; and 2) in the colon [*CD2* (Rh02839718_m1), *CCL19*

(Rh02621767_m1), *STAT1* (Rh02899274_m1), and *ZAP70* (Rh02837378_m1)] were selected for confirmation using Taqman probes and qRT-PCR. Changes in expression level of all 8 genes were confirmed. Functional enrichment analysis was performed to identify significant biological pathways including gene ontology (GO) terms and disease biomarkers using MetaCore™ software (GeneGo). Since this software requires the use of human gene ID, all rhesus macaque DEG were mapped to human homologs using annotations from ENSEMBL.

The gene expression database for all characterized immune cells in the mouse developed by the Immunological Genome Project (ImmGen) was used to identify which cell types drive gene expression changes [320].

The software program Short Time-series Expression Miner (STEM; v1.3.11) was utilized to further identify clusters of genes that show similar patterns of expression as it relates to discrete doses of ethanol consumption: none, non-heavy, and heavy [321].

16S rRNA gene library construction and sequencing

Total DNA was extracted from duodenal, jejunal, ileal, and colonic biopsies using PowerSoil DNA Isolation kit (MO BIO Laboratories, Inc., Catalog #12888-100). PCR and custom primers were used to amplify the V4-V5 region of the 16S rRNA gene as previously described [322]. Libraries were sequenced (250 bases) using an Illumina MiSeq.

16S rRNA gene bioinformatics analyses

Low quality, chimeric, and Cyanobacteria sequences were removed using Quantitative Insights Into Microbial Ecology (QIIME) [323]. This process resulted in 2,820,185 total reads with per sample counts ranging from 19,579 to 128,131, with an average of 58,754 counts/sample. Reference operational taxonomic units (OTU) were selected at 97% similarity using QIIME and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt). Taxonomic assignments were made using the 2013 Greengenes reference database [324]. OTU with fewer than 100 sequences were removed from additional analyses. QIIME was used to calculate alpha diversity including OTU observed and Shannon diversity indices, beta diversity UniFrac values, and perform Adonis analyses. PICRUSt was used to adjust OTU abundances for their rRNA copy number and impute the predicted metagenome of the bacterial communities based on the 16S rRNA gene sequences [325]. Spearman correlation analyses were performed in QIIME between 12-month average BEC and either the OTU or the Kyoto Encyclopedia of Genes and Genomes (KEGG) K numbers generated by the PICRUSt analyses. Correlations were defined as those with an r-value >0.5 or <-0.5, and a p-value <0.05. Heatmaps depicting these correlation values were created using custom R scripts and four libraries (ggplot2, ggdendro, reshape2, and grid) [326-329]. The 16S rRNA gene sequence data have been deposited in NCBI's SRA under the accession number SUB1983942 (http://www.ncbi.nlm.nih.gov/sra).

Determination of anti-endotoxin IgM antibodies

Endotoxin-core antibodies in plasma samples were measured using an enzyme-immunoassay technique (ELISA) after 12 months of alcohol consumption using EndoCab IgM ELISA kit (Hycult Biotech, Catalog# HK504-IgM). Plasma samples were diluted 50x.

Statistical analysis

Statistical significance of caloric intake, IgM, ALT, AST, values between controls, non-heavy drinkers, and heavy drinkers was assessed with a one-way ANOVA followed by Bonferroni's multiple comparison correction tests. Spearman correlation analyses were performed to define associations between anti-endotoxin IgM antibody titer and average 12-month BEC or daily g/kg ethanol consumed. Expression data for each Taqman probe was calculated relative to control RPL32 mRNA expression using ΔCt calculations and statistical significance was assessed using an unpaired T-test. These analyses were carried out by GraphPad Prism version 6 (GraphPad software).

Availability of data and material

The RNA-Seq data have been deposited in NCBI's Sequence Read Archive (SRA) under the accession number SUB1885356 (http://www.ncbi.nlm.nih.gov/sra). The 16S rRNA gene sequence data have been deposited in NCBI's SRA under the accession number SUB1983942 (http://www.ncbi.nlm.nih.gov/sra).

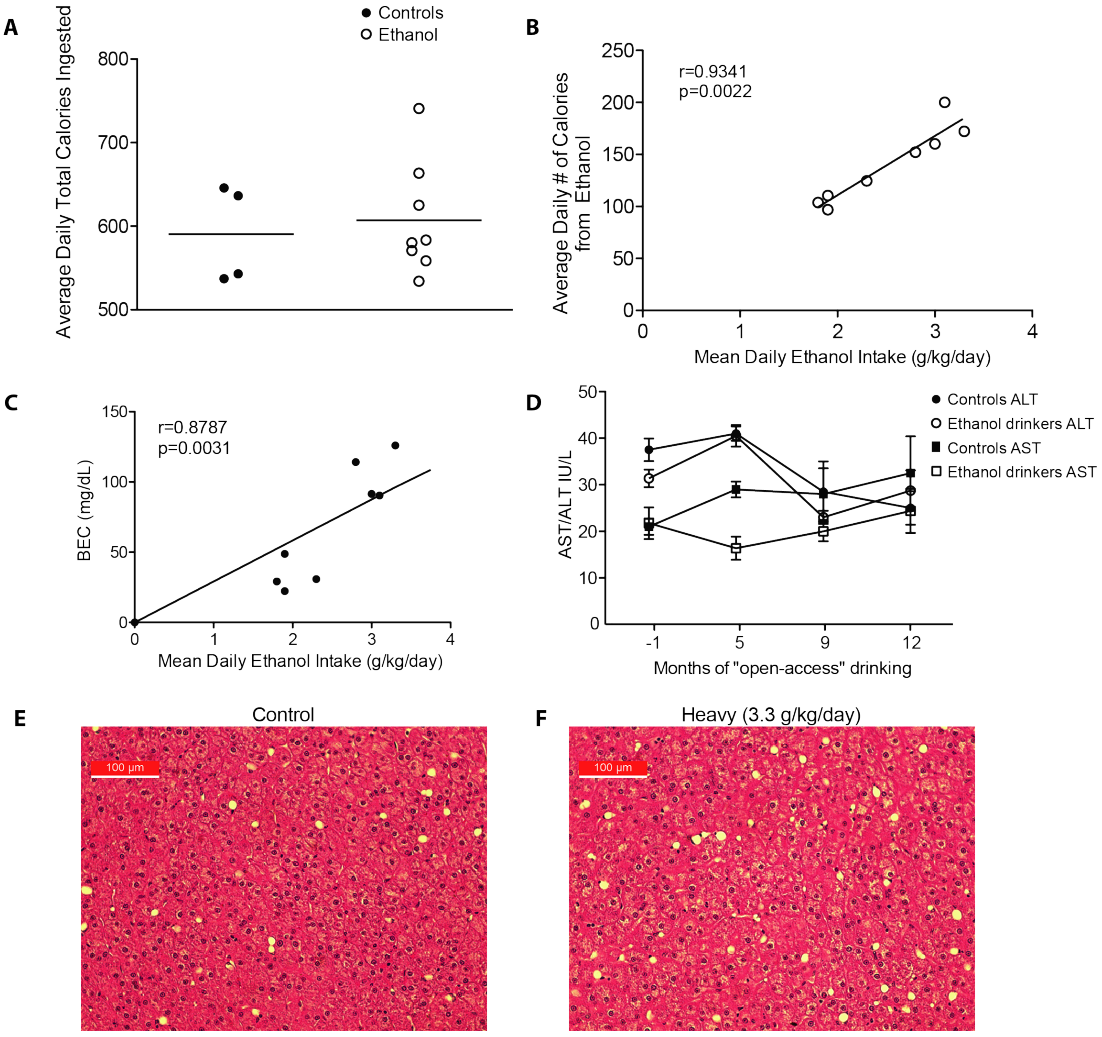


Figure 3.1: Characteristics of macaques used in these studies. Scatterplots showing the average daily number of (A) total calories ingested from either the calorically-matched maltose-dextrose solution or ethanol-consuming male macaques. (B) Correlation plot between average daily number of calories from ethanol versus the mean daily ethanol intake (g/kg/day). (C) 12-month average blood ethanol concentration (BEC) and g/kg/day consumed strongly correlate with one another. (D) Line graphs of average ± SEM ALT (normal range = 18.9-94.2) and AST (normal range = 18-58) values in controls and ethanol-consuming animals. Statistical analysis was assessed with a two-way ANOVA and shows that ALT and AST levels are comparable between all three groups. (E, F) Histological assessment shows lack of liver injury in the male macaques used in these studies.

Results

Chronic ethanol consumption modulates expression of genes critical for regulation of gene expression, metabolism, and cell adhesion in the jejunum

Although it is believed most of the ethanol consumed is rapidly absorbed into circulation in the duodenum, we did not detect any differentially expressed genes (DEG) in the duodenal biopsies. In the jejunum, we detected 45 DEG between controls and ethanol-consuming male macaques (Fig. 3.2A). The 28 DEG with human homologs (Fig. 3.2B) play a role in the regulation of gene expression, protein trafficking, and metabolism (Fig. 3.2C). Upregulated DEG that play a role in regulating gene expression include DNA replication licensing factor (*MCM6*), histone H3 (*H3F3A*), and serine/arginine-rich splicing factor (*SRSF11*). Upregulated DEG that are important for protein trafficking include zinc finger DHHC-type-containing-6 (*ZDHHC6*; protein folding) and inner mitochondrial membrane peptidase-like-1 (*IMMP1L*; protein export). Finally, upregulated DEG with a role in cellular metabolism include those involved in digestion of carbohydrates such as sucrose-isomaltase (*SI*) and enolase-4 (*ENO4*), as well as fatty acid metabolism such as acyl-coenzyme-A synthetase medium chain-3 (*ACSM3*) and apolipoprotein B (*APOB*). Several of the downregulated DEG also played a role in regulation of: gene expression such as chromatin-modifying protein-1A (*CHMP1A*); metabolism such as the insulin-induced gene-2 (*INSIG2*); and cell movement such as the adhesion molecule *CD109*.

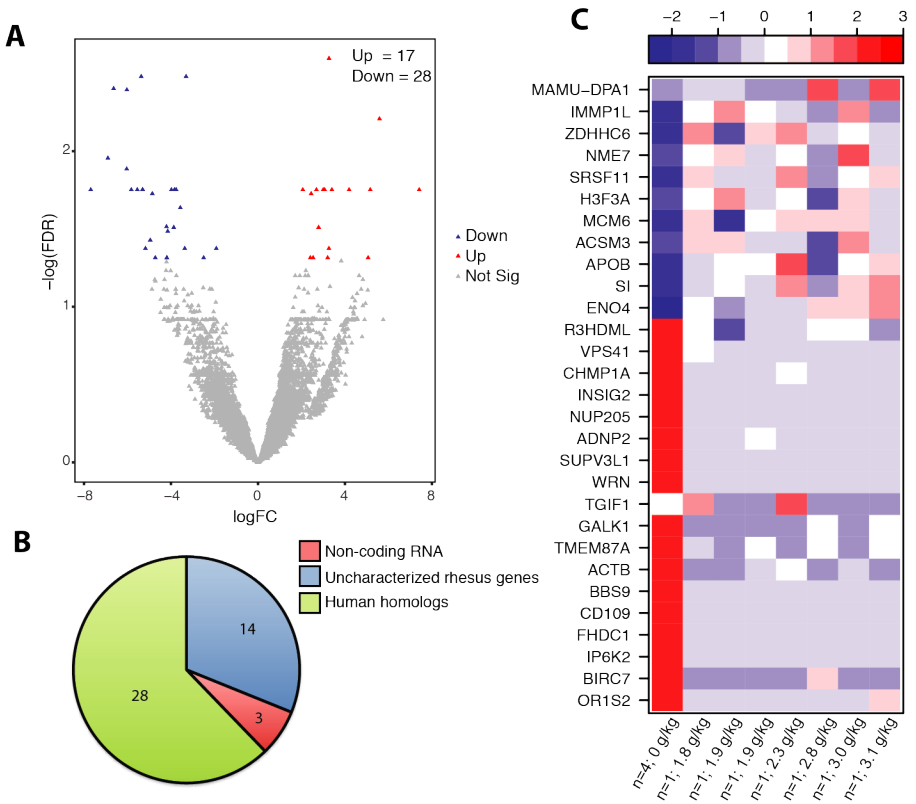


Figure 3.2: Chronic ethanol consumption modulates jejunum mucosal gene expression. DEG were identified using the generalized linear model likelihood ratio test method from the *edgeR* package as those with a fold change of ≥2 and a Benjamini-Hochberg- corrected false discovery rate (FDR) of <0.05. (A) Volcano plot summarizing the gene expression changes with red representing the upregulated DEG and blue representing the downregulated DEG. The number of up- and down-regulated genes is noted. (B) Pie chart representing the breakdown of DEG as: noncoding RNA, uncharacterized rhesus genes, and human homologs. (C) Heatmap representing gene expression (shown as absolute normalized RPKM values) of the genes listed; first column shows median RPKM values of the controls (n=4), and subsequent columns show RPKM values of each individual ethanol-consuming animals ordered by alcohol dose (g of ethanol/kg/day); range of colors is based on scaled and centered RPKM values of the entire set of genes (red represents increased expression while blue represents decreased expression).

Chronic ethanol consumption modulates expression of genes important for immune processes in the ileum

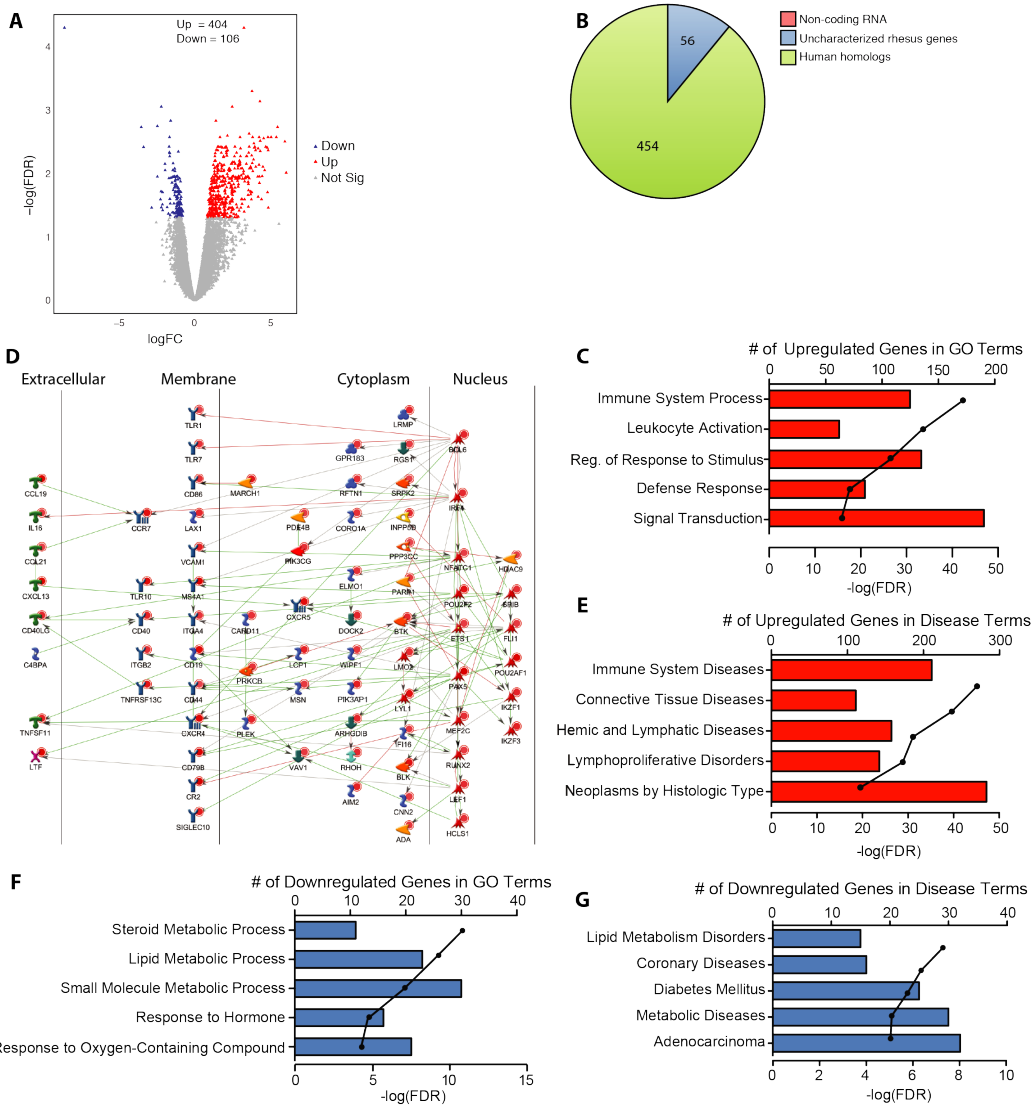
The ileum exhibited the most robust changes in gene expression with 510 DEG (Fig. 3.3A). Of the 454 DEG with human homologs (Fig. 3.3B), 363 were upregulated and 91 were downregulated. The upregulated DEG primarily enriched to gene ontology (GO) terms related to immunity and signal transduction (Fig. 3.3C). A nework analysis using the functional enrichment software Metacore™ showed that of the 136 upregulated DEG that functionally enriched to the GO process "immune system process", 73 DEG directly interact with one another (Fig. 3.3D). These genes include T-cell markers (*CD1C*, *CD40LG*); B-cell markers (*CD19*, *CD79B*); antigen presenting cell markers (*CD40*, *CD86*); adhesion molecules (*VCAM1*, *CD44*); cytokines, chemokines, and their receptors (*CCL19*, *CCR6*, *CCR7*, *TNFRSF13C*); and Toll-like receptors (*TLR1*, *TLR7*, *TLR10*). The majority of the DEG that functionally enriched to "signal transduction" also enriched to "immune system process". Those that uniquely enriched to "signal transduction" play a role in G-protein signaling e.g., regulator of G protein signaling *RGS13*, the signaling G protein *RAC2*, the guanine nucleotide exchange factor GIV/Girdin (*CCDC88A*), and the GTPase dynamin (*DNM1*).

Additional functional enrichment analysis using a disease database available on Metacore™ revealed significant enrichment to immune system diseases (Fig. 3.3E) including genes that are critical to the development and progression of various lymphomas such as *BCL2A1*, *BCL6*, Bruton's tyrosine kinase (*BTK*), B- and T-lymphocyte attenuator (*BTLA*), matrix metalloproteinases (*MMP12*), and mitogen-activated protein kinases

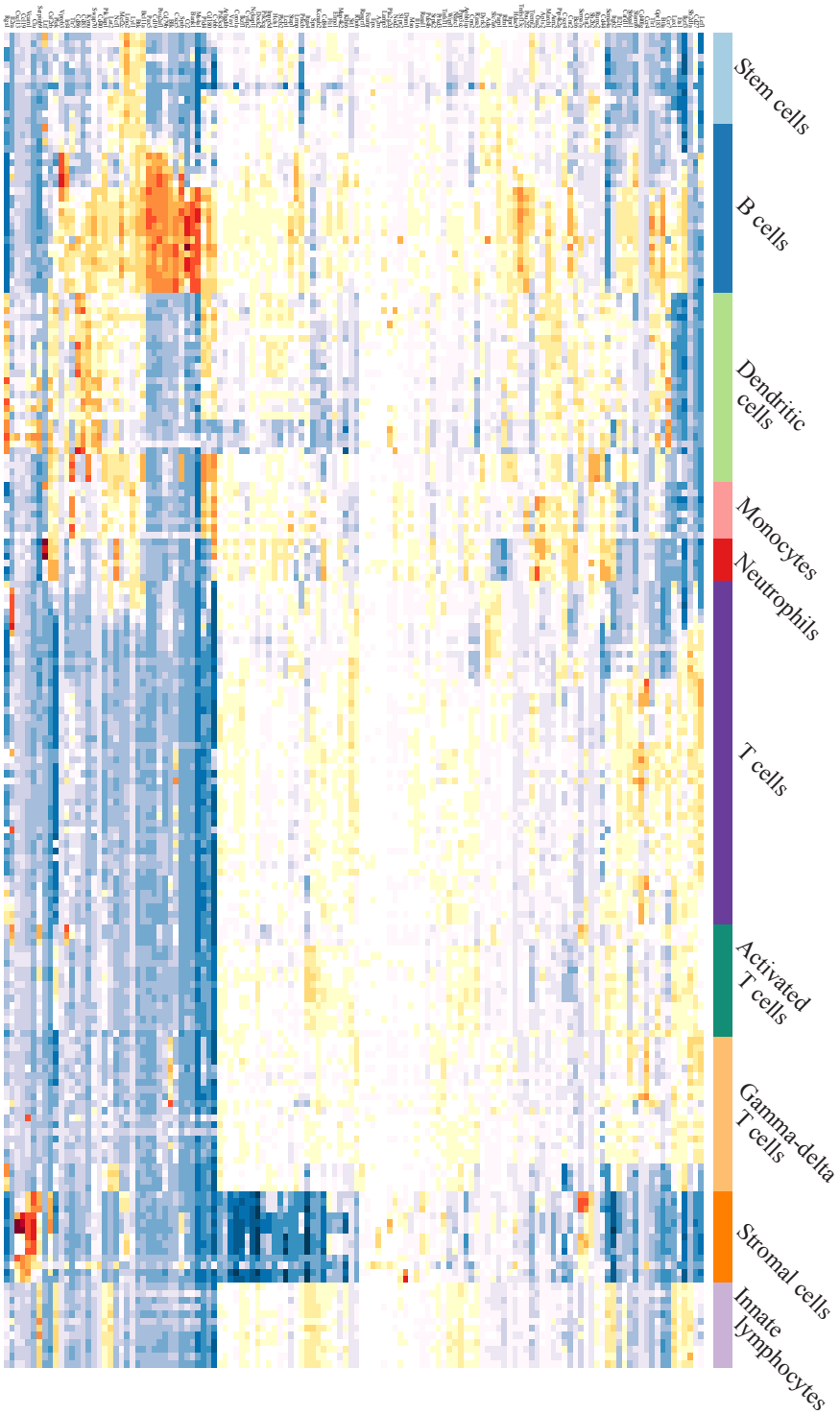
(*MAP4K1*). Most of the 108 DEG that uniquely enriched to "neoplasms by histologic type" are associated with GI neoplasms including complement component-4 binding protein alpha (*C4BPA)*, protein tyrosine phosphatase non-receptor type-7 (*PTPN7)*, and transcription factor 4 (*TCF4)*. Expression of genes involved in structural integrity such as gap junction protein (*GJA5*) was also upregulated.

The 91 DEG that were downregulated in the ileum with ethanol consumption enriched to GO processes related to steroid/lipid metabolism (Fig. 3.3F). Notable DEG include the signaling molecule phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type-2-gamma (*PIK3C2G*); transcription factors hepatocyte nuclear factor alpha 1 and 4 (*HNFA1*, *HNFA4*); and the low-density lipoprotein receptor (*LDLR*) and its ligand proprotein convertase subtilisin/kexin type-9 (*PCSK9)*. Similarly, analysis using the diseases database showed enrichment to "lipid metabolism disorders" and "metabolic diseases". Additionally, several of the 32 genes that enriched to the disease pathway "adenocarcinoma" (Fig. 3.3G) are associated with progression of CRC including caspase recruitment domain-containing protein (*PYCARD*) and suppressor of cytokine signaling-6 (*SOCS6*).

In order to determine the likely source of these immune gene expression changes, we imputed the 136 upregulated DEGs that enriched to the GO term "immune system process" into the gene expression database ran by the Immunological Genome Project (ImmGen) [320]. This *in silico* analysis revealed the majority of gene expression changes associated with immunity are derived from B lymphocytes, neutrophils, and stromal cells (Fig. 3.4).


A
Up = 404
Down = 106
Down
Up
Not Sig
-log(FDR)
logFC
B
56
454
Non-coding RNA
Uncharacterized rhesus genes
Human homologs
C
# of Upregulated Genes in GO Terms
Immune System Process
Leukocyte Activation
Reg. of Response to Stimulus
Defense Response
Signal Transduction
-log(FDR)
D
Extracellular
Membrane
Cytoplasm
Nucleus
E
# of Upregulated Genes in Disease Terms
Immune System Diseases
Connective Tissue Diseases
Hemic and Lymphatic Diseases
Lymphoproliferative Disorders
Neoplasms by Histologic Type
-log(FDR)
F
# of Downregulated Genes in GO Terms
Steroid Metabolic Process
Lipid Metabolic Process
Small Molecule Metabolic Process
Response to Hormone
Response to Oxygen-Containing Compound
-log(FDR)
G
# of Downregulated Genes in Disease Terms
Lipid Metabolism Disorders
Coronary Diseases
Diabetes Mellitus
Metabolic Diseases
Adenocarcinoma
-log(FDR)

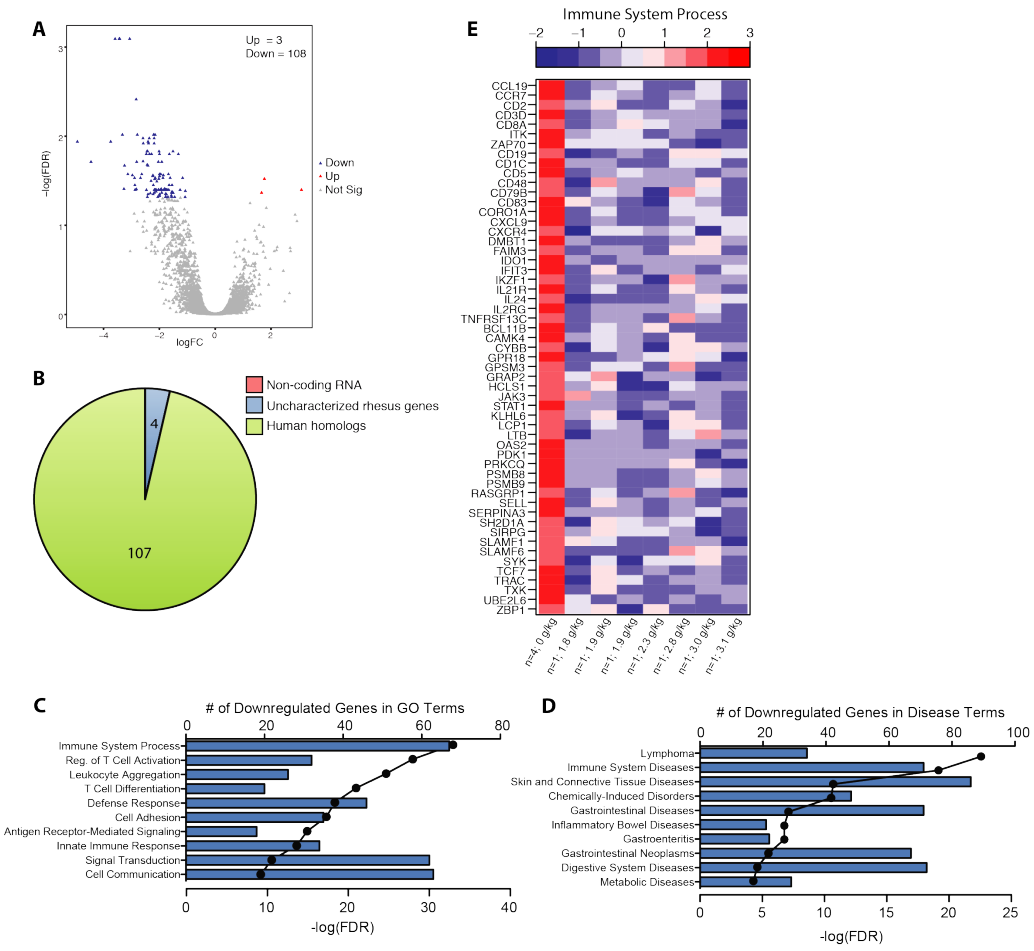
(Previous page) Figure 3.3: Chronic heavy ethanol consumption modulates expression of genes important for immune system processes in the ileum. DEG were identified using the generalized linear model likelihood ratio test method from the *edgeR* package as those with a fold change of ≥2 and a Benjamini-Hochberg- corrected false discovery rate (FDR) of <0.05. (A) Volcano plot summarizing the gene expression changes with red representing the upregulated DEG and blue representing the downregulated DEG. The number of up- and down-regulated genes is noted. (B) Pie chart representing the breakdown of DEG as noncoding RNA, uncharacterized rhesus genes, and human homologs. (C) Bar graphs displaying the number of DEG upregulated with ethanol consumption that enriched to the listed Gene Ontology (GO) terms; line represents the -log(FDR-adjusted P-values) associated with each enrichment to a GO term. (D) Network image of the upregulated DEG that enriched to "immune system process" and directly interact with one another. (E) Bar graphs displaying the number of DEG upregulated with ethanol consumption that enriched to the Disease terms; line represents the -log(FDR-adjusted P-values) associated with each Disease. (F) Bar graphs displaying the number of DEG downregulated with ethanol consumption that enriched to the listed GO terms; line represents the -log(FDR-adjusted P-values) associated with enrichment to each GO term. (G) Bar graphs displaying the number of DEG upregulated with ethanol consumption that enriched to the Disease terms; line represents the -log(FDR-adjusted P-values) associated with each Disease term.


Stem cells
B cells
Dendritic cells
Monocytes
Neutrophils
T cells
Activated T cells
Gamma-delta T cells
Stromal cells
Innate lymphocytes

(Previous page) Figure 3.4: *In silico* analysis shows B cells drive most gene expression changes associated with immunity. Heatmap representing gene expression (shown as absolute normalized RPKM values) of the upregulated ileal genes that enriched to the GO term "immune system process" and have mouse homologs detected by ImmGen. Range of colors is based on scaled and centered RPKM values of the entire set of genes (red represents increased expression while blue represents decreased expression) and specific type of immune cells driving these changes in expression are noted.

Chronic ethanol consumption modulates expression of genes important for pathways involving cancer development and immunity in the colon mucosa

In the colon, 3 genes were upregulated and 108 genes were downregulated with ethanol-consumption (Fig. 3.5A). Of the 107 downregulated DEG with human homologs (Fig. 3.5B), 67 enriched to the GO term "immune system process" (Fig. 3.5C and E) and 71 enriched to the disease term "immune system diseases" (Fig. 3.5D). These genes include B-cell markers (*CD19*, *CD72*, *CD79B*); co-stimulatory markers (*CD83*); as well as cytokines, chemokines, and their receptors (e.g. *IL24*, *CCL19*, *IL21R*, and *CCR7*). Several DEG also encode for T-cell markers (e.g. *CD1C*, *CD2*, *CD3D*, *CD3E*, *CD5*, *CD8A*) and enriched to GO terms "regulation of T-cell activation" and "T-cell differentiation". Interestingly, several of these DEG were upregulated with ethanol consumption in the ileum (*CD1C*, *CD19*, *CD22*, *CD72*, *CD79B*, *CCL19*, *CCR7*, *CXCR4*, *FCRL1*, *IL21R*, *TNFRSF13C)*. Additional downregulated immune genes include indoleamine-2,3-dioxygenase-1 (*IDO1*), which drives differentiation of regulatory T-cells; peptidase inhibitor (*PI3*); and defensin-6 (*DEF6*). Some down-regulated genes are also critical for signal transduction including Janus kinase-3 (*JAK3*) and signal transducer and activator of transcription (*STAT1*). Several of the immune genes listed above enriched to the disease term "gastrointestinal neoplasms" and are also associated with CRC development including the T cell chemoattractant *CXCL9*, keratin-7 (*KRT7)*, and *IDO1*.


A
Up = 3
Down = 108
−log(FDR)
logFC
Down
Up
Not Sig
B
Non-coding RNA
Uncharacterized rhesus genes
Human homologs
4
107
E
Immune System Process
−2 −1 0 1 2 3
CCL19
CCR7
CD2
CD3D
CD8A
ITK
ZAP70
CD19
CD1C
CD5
CD48
CD79B
CD83
CORO1A
CXCL9
CXCR4
DMBT1
FAIM3
IDO1
IFIT3
IKZF1
IL21R
IL24
IL2RG
TNFRSF13C
BCL11B
CAMK4
CYBB
GPR18
GPSM3
GRAP2
HCLS1
JAK3
STAT1
KLHL6
LCP1
LTB
OAS2
PDK1
PRKCQ
PSMB8
PSMB9
RASGRP1
SELL
SERPINA3
SH2D1A
SIRPG
SLAMF1
SLAMF6
SYK
TCF7
TRAC
TXK
UBE2L6
ZBP1
n=4, 0 g/kg
n=1, 1.8 g/kg
n=1, 1.9 g/kg
n=1, 1.9 g/kg
n=1, 2.3 g/kg
n=1, 2.8 g/kg
n=1, 3.0 g/kg
n=1, 3.1 g/kg
C
# of Downregulated Genes in GO Terms
Immune System Process
Reg. of T Cell Activation
Leukocyte Aggregation
T Cell Differentiation
Defense Response
Cell Adhesion
Antigen Receptor-Mediated Signaling
Innate Immune Response
Signal Transduction
Cell Communication
-log(FDR)
D
# of Downregulated Genes in Disease Terms
Lymphoma
Immune System Diseases
Skin and Connective Tissue Diseases
Chemically-Induced Disorders
Gastrointestinal Diseases
Inflammatory Bowel Diseases
Gastroenteritis
Gastrointestinal Neoplasms
Digestive System Diseases
Metabolic Diseases
-log(FDR)

(Previous page) Figure 3.5: Chronic heavy ethanol consumption modulates expression of genes associated with cancer development and immune function in the colon. DEG were identified using the generalized linear model likelihood ratio test method from the *edgeR* package as those with a fold change of ≥2 and a Benjamini-Hochberg-corrected false discovery rate (FDR) of <0.05. n=4/group. (A) Volcano plot summarizing the gene expression changes with red representing the upregulated DEG and blue representing the downregulated DEG. The number of up- and down-regulated genes is noted. (B) Pie chart representing the breakdown of DEG as noncoding RNA, uncharacterized rhesus genes, and human homologs. (C, D) Bar graphs displaying the number of DEG downregulated with ethanol consumption that enriched to GO and Disease terms respectively; line represents the -log(FDR-adjusted P-values) associated with enrichment to each GO/disease term. (E) Heatmap representing gene expression (shown as absolute normalized RPKM values) of DEG that enriched to the GO term "immune system process"; first column shows median RPKM values of the controls (n=4), and subsequent columns show RPKM values of each individual ethanol-consuming animals ordered by alcohol dose (g of ethanol/kg/day); range of colors is based on scaled and centered RPKM values of the entire set of genes (red represents increased expression while blue represents decreased expression).

Ethanol consumption results in dynamic dose-dependent mucosal gene expression changes

Since we collected samples from male macaques with varying ethanol intake, we next explored dose-dependent dynamic changes in gene expression that go beyond positive or negative correlations by utilizing Short Time-series Expression Miner (STEM). Animals were divided into 3 groups based on their ethanol consumption patterns: controls (n=4), non-heavy drinkers (n=4), and heavy drinkers (n=4) [34]. We focused our analysis on highly expressed genes with an average RPKM value ≥50 (reads per kilobase of transcript per million mapped reads). In the duodenum, STEM analysis identified 2 clusters of genes the expression of which increases with non-heavy ethanol consumption then decreases to either control or near control levels with heavy drinking. Genes in cluster 1 play a role in protein transport and localization (Fig. 3.6A and Fig. 3.7A) and functionally enriched to GO terms such as "cotranslational protein targeting to membrane" (FDR-P=1.15e-17; e.g ribosomal proteins *RPL11* and *RPS27*) and "protein targeting to ER" (FDR-P=2.66e-17). Genes also enriched to "transport" (FDR-P=1.30e-10; e.g. solute carrier *SLC26A3*) and "immune response-regulating signaling pathway" (FDR-P=8.11e-7; e.g. immunoglobulin genes *IGHM* and *IGJ*). Furthermore, 41 of these 47 genes enriched to the Disease term "digestive system diseases" (FDR-P=4.95e-5) including those encoding for the immunoglobulin receptor *PIGR* and the mucin gene *MUC6*, which modulates the composition of the protective mucus layer. Genes in cluster 2 (Fig. 3.6A) play a role in exocytosis and enriched to GO terms "secretion" (FDR-P=1.92e-4) and "neutrophil

degranulation" (FDR-P=1.92e-4) such as heat shock protein *HSPA8*. All 13 genes within cluster 2 also enriched to "colonic diseases" (FDR-P=3.00e-4).

Similarly, in the jejunum, STEM analysis revealed a cluster of genes whose expression increases with non-heavy ethanol consumption then returns to near control levels with heavy ethanol consumption (Fig. 3.6B). Majority of these 129 genes are important for cellular metabolism, and functionally enriched to the GO term "cellular metabolic process" (n=80; FDR-P=5.16e-3) including the Acetyl-CoA Acetyltransferase 1 *ACAT1* and the ubiquitin ligase *MARCH7*. STEM also identified a second group of genes with expression that increases with non-heavy ethanol consumption and remains unchanged with heavy ethanol consumption (Fig. 3.6B) that also functionally enriched to GO term "metabolic process" (FDR-P=8.42e-2; Fig. 3.7B) including the phosphofructokinase *PFKM* and the solute carrier *SLC7A7*. STEM further identified a third cluster of genes in the jejunum, with expression levels that drastically increase with non-heavy ethanol consumption then continue to increase with heavy ethanol consumption (Fig. 3.6B). These genes are critical for signal transduction and include natural killer cell receptor *CD160* and the choline phosphotransferase *CEPT1*.

STEM analysis of the ileum transcriptome uncovered a cluster of 89 genes the expression of which decreases with non-heavy ethanol consumption and to a lesser extent with heavy ethanol consumption (FDR-P=2.3e-20; Fig. 3.6C). These genes functionally enriched to GO terms "neutrophil mediated immunity" (FDR-P=9.25e-6; e.g. chemokine *CCL25* and innate immune gene *PLAC8*), "transport" (FDR-P=9.25e-6; members of the solute carriers family *SCL2, 5, 6 and* 7), and "digestion" (FDR-P=1.16e-5; mucin gene

MUC2 and actin-binding protein *VIL1*). STEM analysis identified a second cluster of 60 genes the expression of which was unaltered with non-heavy ethanol consumption but greatly increased with heavy ethanol consumption (Fig. 3.6C). These genes enriched to the GO term "negative regulation of metabolic process" (FDR-P=1.88e-25; Fig. 3.7C) including heat shock protein *HSP90AB1* and ribosomal protein *UBA52*. Additionally, 38 other genes enriched to the Disease term "colonic diseases" (FDR-P=1.00e-5) such as metastasis suppressor *CD82*, putative tumor suppressor *TAGLN2*, and actin-sequestering *TMSB4X*.

In the colon, STEM analysis identified 37 genes, the expression of which greatly decreased with non-heavy ethanol consumption, and even more with heavy ethanol consumption (Fig. 3.6D). These genes play a role in macromolecule transport and functionally enriched to GO terms such as "protein targeting" (FDR-P=4.67e-20) and "macromolecule localization" (FDR-P=6.20e-11) including cofilin gene *CFL1* and carbohydrate binding galectin 3 *LGALS3*. Furthermore, 32 of these genes enriched to the Disease term "gastrointestinal neoplasms" (FDR-P=1.88e-3) including gut permeability indicator *FABP* and signaling molecule *RACK1*. STEM identified an additional cluster of genes where expression negatively correlated with increasing ethanol dose in a linear fashion (Fig. 3.6D and Fig. 3.7D). Functional enrichment showed that 16 genes are associated with "localization" (FDR-P=7.78e-12) and 19 of these genes mapped to the Disease term "colonic diseases" (FDR-P=3.17e-4), notably adhesion molecule *CD9*, and components of the actin cytoskeleton such as *ACTB*.

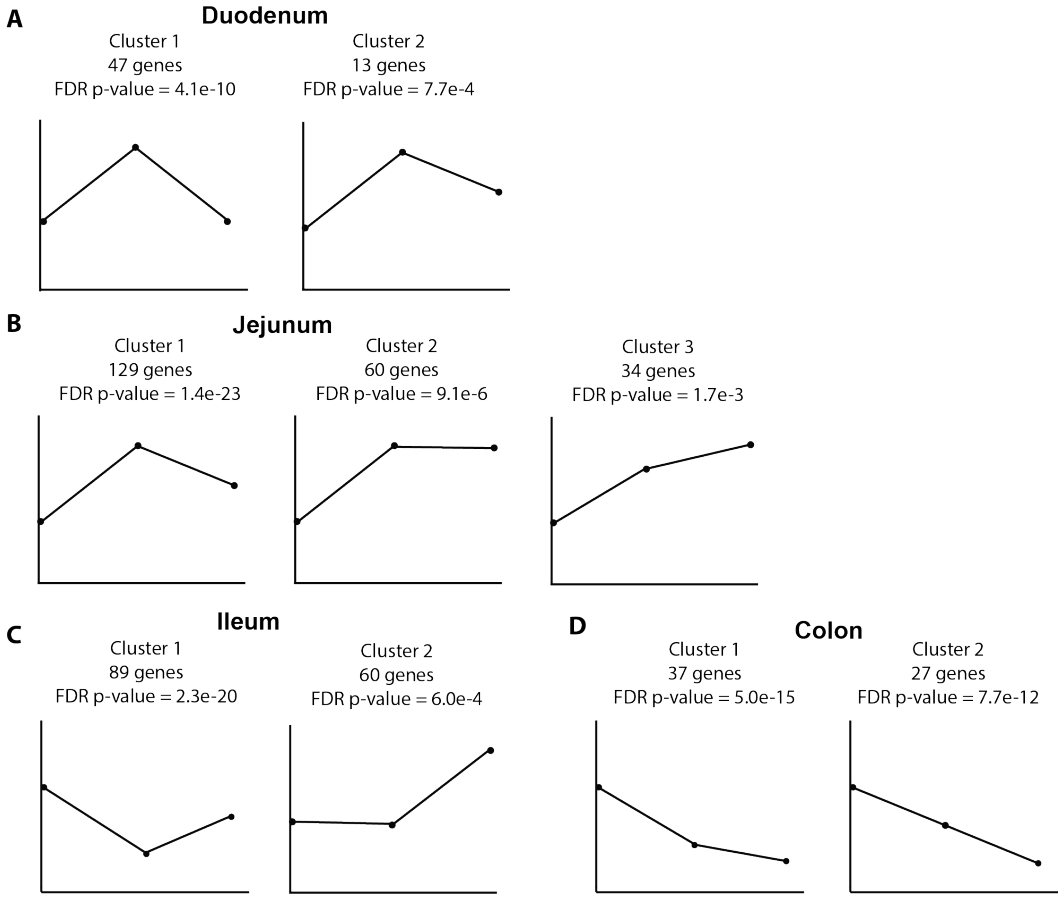


Figure 3.6: Dynamic gene expression changes mediated by ethanol consumption. Expression patterns of DEG clusters uncovered by STEM analysis in (A) duodenum, (B) jejunum, (C) ileum, (D) and colon with the number of genes and FDR p-value reported. First point represents controls (n=4); second point represents non-heavy group (n=4) and last point represents heavy group (n=4).

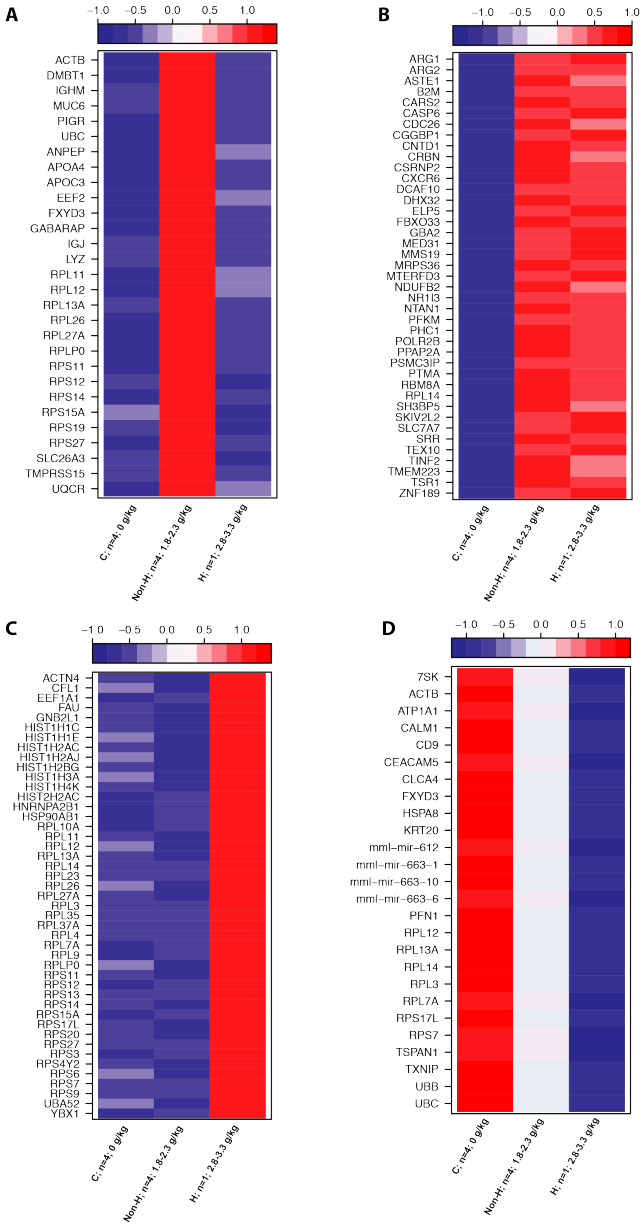


Figure 3.7: STEM gene expression patterns. Heatmap showing relative median expression levels in controls (C), non-heavy drinkers (Non-H), and heavy drinkers (H) of genes in: (A) duodenum cluster 1 that enriched to "transport" and "immune response-regulating signaling pathway"; (B) jejunum cluster 2 that enriched to "metabolic process"; (C) ileum cluster 2 that enriched to "negative regulation of metabolic process"; and (D) colon cluster 2.

Given the robust changes in gene expression observed along the GI tract, we next determined if these alterations were accompanied by an increased permeability of the GI barrier. We measured circulating endotoxin-core antibodies in plasma samples collected after 12 months of alcohol consumption and discovered a positive correlation between levels of anti-endotoxin IgM levels and ethanol intake (r=0.8201; p=0.0016) (Fig. 3.8).

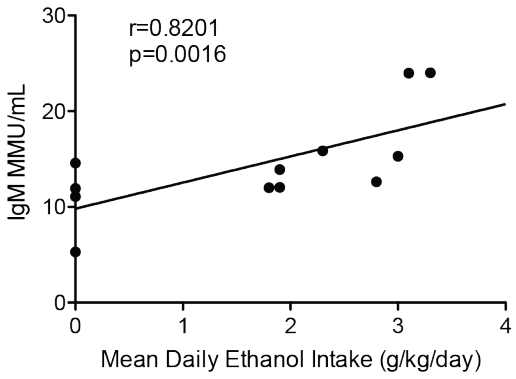


Figure 3.8: Dose-dependent increase in intestinal permeability. Endotoxin-core IgM antibody levels significantly correlate with the 12-month mean daily ethanol.

Alcohol consumption impacts mucosa-associated bacterial communities in both small and large intestine

We next investigated ethanol-induced changes in gut mucosa-associated bacterial communities using 16S rRNA gene sequencing [330]. Since we did not have access to luminal contents, we sequenced the second half of the mucosal biopsies used for RNA-Seq analysis. Although not ideal, this approach would provide insight into the microbial niche that is closely interacting with intestinal epithelial and immune cells. To assess alpha diversity, we calculated the Shannon diversity index, which revealed similarities in evenness among the intestinal regions and groups (Fig. 3.9).

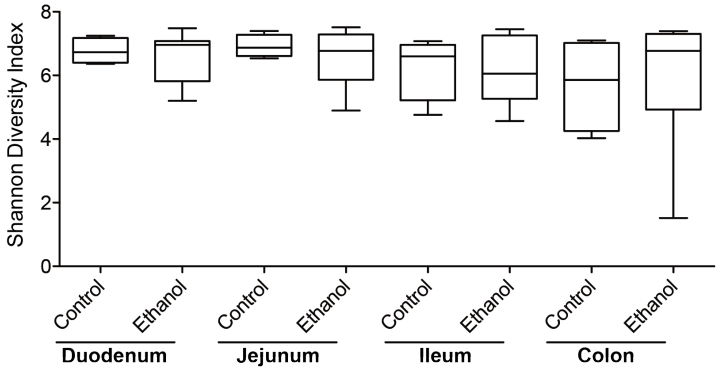


Figure 3.9: Alpha diversity along the gastrointestinal tract. Box plot of Shannon diversity index values from duodenum, jejunum, ileum, and colon between controls and ethanol-consuming male macaques. Statistical analysis was assessed with a one-way ANOVA.

The beta diversity analysis using weighted UniFrac values showed that biopsies from ethanol-consuming male macaques did not form a distinct cluster from those collected from controls (Fig. 3.10A to D). In line with these observations, only subtle differences were noted at the phyla level (Fig. 3.11A and B). In the jejunum and ileum, we observed a small decrease in relative abundance of *Firmicutes* in ethanol-consuming animals compared to controls (36.3% and 36.8% compared to 41.2% and 42.8%). In the colon, we detected a slight increase in *Bacteroidetes* (25.2% vs. 18.2%) that was accompanied by a decrease in *Proteobacteria* (33.2% vs. 38.3%) in ethanol-consumers compared to controls (Fig. 3.11A). A genus level analysis further revealed minimal changes in the colon, where levels of *Flexispira* slightly decreased while Clostridiales, S24-7, and *Prevotella* increased with ethanol consumption (Fig. 3.12).

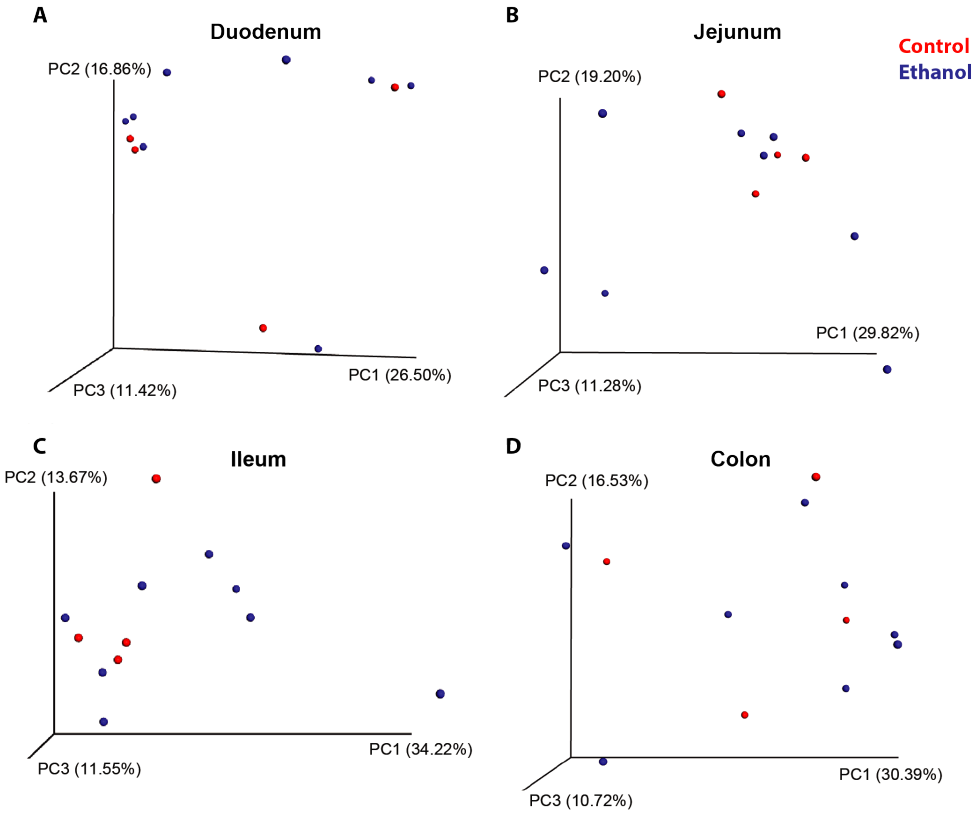
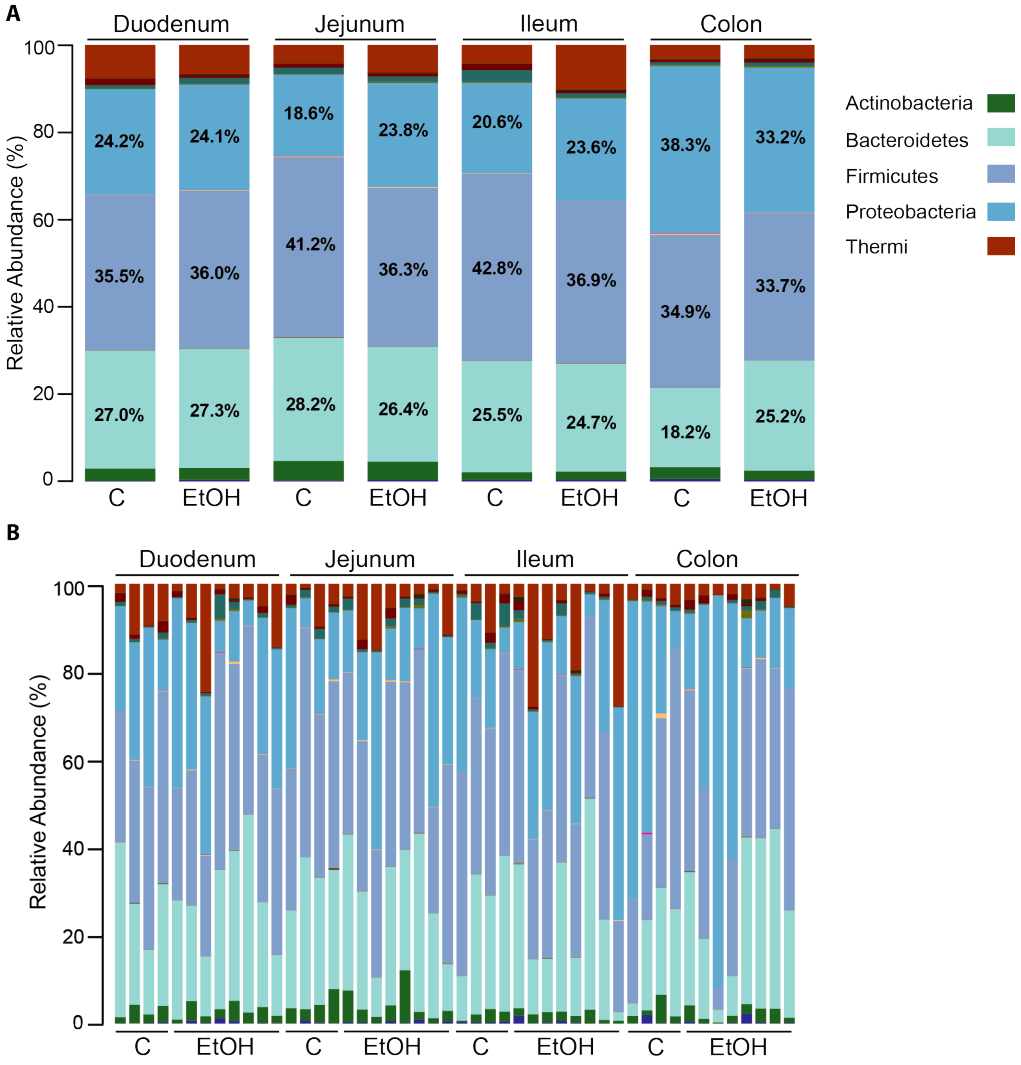


Figure 3.10: Beta diversity along the gastrointestinal tract. PCAs showing beta diversity within (A) duodenum, (B) jejunum, (C) ileum, and (D) colon biopsies.



<u>Figure 3.11</u>: Chronic ethanol intake results in minor changes in mucosal microbial communities. Low quality, chimeric, and Cyanobacteria sequences were removed, reference OTU were selected at 97% similarity, and beta diversity calculations were performed using QIIME. n=4/group. (A) The average and (B) individual abundance (% of total) of bacterial rRNA gene sequences at the phylum level in the duodenum, jejunum, ileum, and colon of controls and ethanol-consuming male macaques. The % abundance of Bacteroidetes, Firmicutes, and Proteobacteria are noted on the plots.

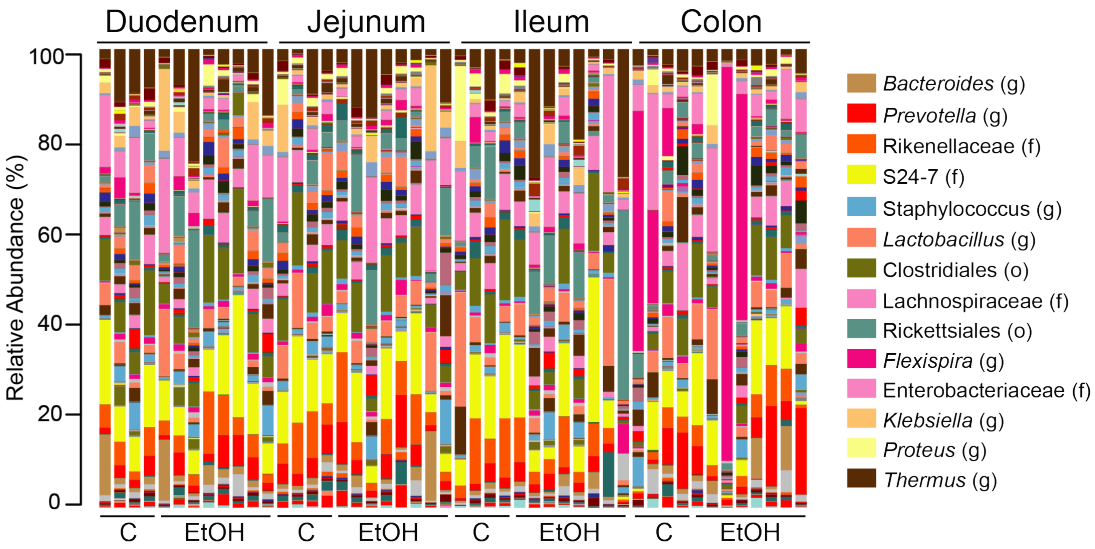


Figure 3.12: Inter-individual family differences. The relative abundance (% of total) of bacterial rRNA gene sequences at the genus level in the duodenum, jejunum, ileum, and colon of each control and ethanol-consuming male macaque ordered by alcohol dose (average daily g of ethanol/kg).

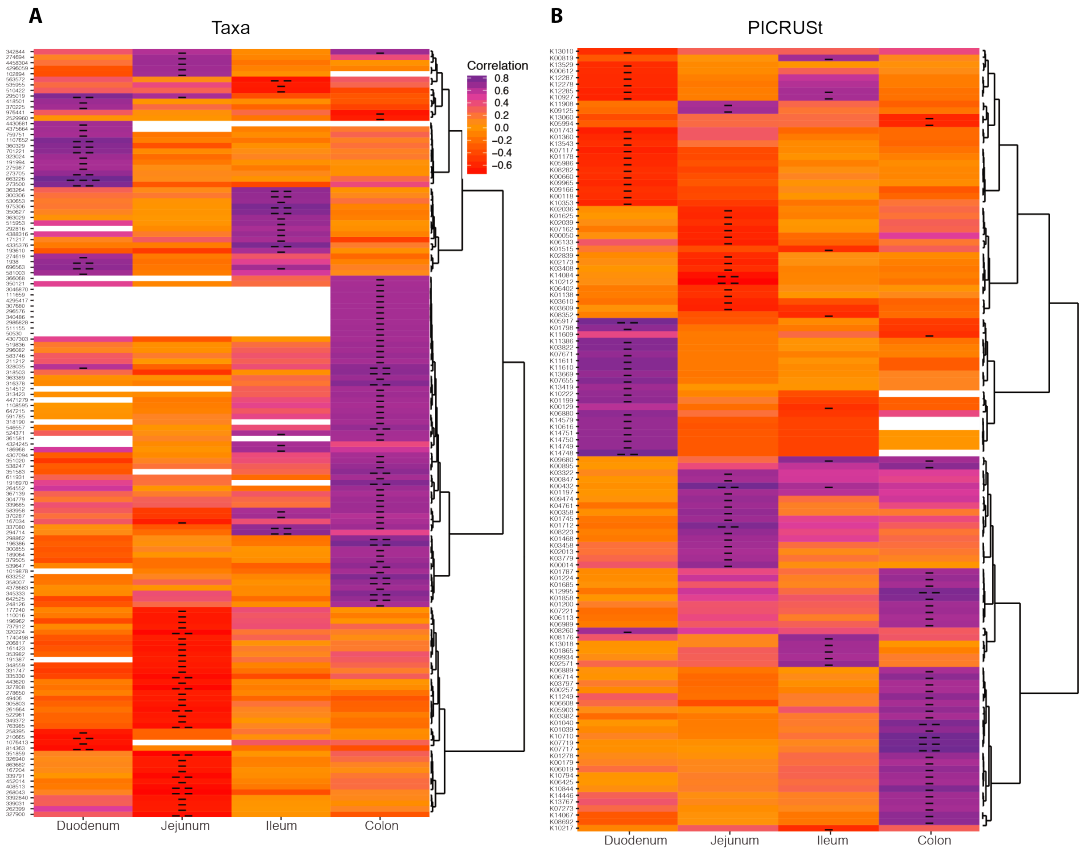
Since we did not detect significant differences in microbial communities at the group level, we decided to examine Spearman correlations between individual average daily ethanol consumption and operational taxonomic unit (OTU) abundance. We also utilized Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to carry out an *in silco* analysis of the pathways associated with the bacterial communities using K numbers from the KEGG database and measured correlations with ethanol consumption. In the duodenum, these analyses revealed 19 OTU whose abundance positively correlated with ethanol consumption including several members of the *Porphyromonadaceae* (Fig. 3.13A). Only 4 OTU, which were members of the *Proteobacteria* and *Bacteroidetes* phyla, negatively correlated with ethanol consumption in the duodenum (Fig. 3.13A). PICRUSt analysis revealed 21 putative bacterial genes associated with the metabolism of carbon-compounds, carbohydrates, and lipids that positively correlated with ethanol consumption (Fig. 3.13B). An additional putative 19 bacterial genes involved in metabolism of purines and carbohydrates negatively correlated with ethanol consumption (Fig. 3.13B).

In the jejunum, 4 OTU positively correlated with ethanol consumption including the opportunistic pathogen *Prevotella veroralis* [331]. Many of the 34 OTU that negatively correlated with ethanol consumption were members of *Clostridiales* including OTU in the *Lachnospiraceae* and *Ruminococcaceae* families (Fig. 3.13A). PICRUSt analysis revealed 18 and 16 putative bacterial genes in the jejunum that positively and negatively correlated with ethanol consumption (Fig. 3.13B), including 4'-phosphopantetheinyl transferase,

which negatively correlated with ethanol consumption and is important for the metabolism of cofactors and vitamins.

In the ileum, 19 OTU positively correlated with BEC including several *Prevotella* species (*P. copri*, *P. stercorea*, and *P. loescheii*) that are known to be associated with inflammation (Fig. 3.13A). The 3 OTU that negatively correlated with BEC included likely commensal bacteria such as *Ruminococcus torques*. PICRUSt analysis revealed 14 putative bacterial genes largely involved in sugar and fatty acid synthesis that positively correlated with ethanol consumption while 6 bacterial genes involved in amino acid, sulfur, and purine metabolism were negatively associated with ethanol consumption (Fig. 3.13B).

The most robust changes in bacterial communities following chronic ethanol consumption were detected in the colon where the abundance of 55 OTU positively correlated with ethanol consumption (Fig. 3.13A). These included *Peptostreptococcaceae bacterium*, *Haemophilus parainfluenzae*, and *Selenomonas dianae*, which are enriched in CRC patients [332, 333] and associated with worse prognosis [334]. PICRUSt analysis suggests that these changes in relative OTU abundance could be associated with the upregulation of pathways involving biosynthesis of carbohydrates (glyoxylate metabolism, amino and nucleotide sugar metabolism); xenobiotics metabolism; and nutrient metabolism (Fig. 3.13B). In contrast, PICRUSt analysis indicated beta-ketoacyl-ACP synthase, critical for vitamin synthesis, was negatively correlated with ethanol intake.



<u>Figure 3.13</u>: Chronic ethanol consumption alters bacterial communities and subsequently critical metabolic pathways in a dose-dependent manner. Heatmaps of (A) OTU and (B) KEGG genes that significantly correlated with average daily BEC either in a positive or negative manner as determined by the Spearman correlation coefficient (n=12/gut region). Correlations were defined as those with an r-value ≥0.5 or ≤-0.5, and a p-value <0.05. Single and double hyphens in the cells indicate p <0.05 and p <0.01, respectively.

Discussion

Among the different models used in alcohol research, nonhuman primates are critically important due to their genetic homology with humans, their propensity to voluntarily consume alcohol, and the similarity of their alcohol metabolism to that of humans [169]. Here, we used a nonhuman primate model of voluntary ethanol self-administration to characterize the site- and dose-dependent impact of chronic ethanol consumption on intestinal mucosa gene expression and bacterial composition in male rhesus macaques.

We did not identify any gene expression differences at the group level associated with ethanol intake in the duodenum, possibly due to the rapid ethanol absorption, the scarcity of resident immune cells in this GI segment [115], and/or the wide range of ethanol doses among the ethanol-consuming animals. However, additional analyses that leveraged the variable drinking patterns of the animals revealed that the expression level of 60 genes largely involved in protein localization, notably several ribosomal protein and solute carriers, increases with non-heavy ethanol consumption then returns to levels similar or slightly above control levels.

In the jejunum, a modest number of DEG that play a role in the regulation of gene expression, protein trafficking, and metabolism was identified between controls and ethanol-consuming animals. One of the most upregulated DEG in the jejunum is histone 3 (*H3F3A*), which is heavily phosphorylated and acetylated following acute ethanol administration in the liver of male rats [335]. Future studies will determine whether increased expression of *H3F3A* in the jejunum of male ethanol-consuming macaques is

also accompanied with increased protein expression and post-translational modifications that could in turn regulate gene expression in the jejunum. Similarly, STEM analysis revealed dose-dependent changes in the expression of genes involved in signal transduction, regulation of gene expression, and cellular metabolic processes including: several G-coupled protein receptors (*GPR18, 22, 34, and 82*), potassium channel famil y members (*KCNIP4* and *KCNJ13*), transcription factors (*EIF2B1*, *EIF2S2*, and *EIF4A1*), and interestingly microRNA precursors (miR1-2, 133A, 181A, and 215). Collectively, these gene expression changes suggest that ethanol consumption disrupts metabolism, protein trafficking, and regulation of gene expression in the jejunum.

The most robust changes in gene expression at the group level were detected in the ileum, which harbors the majority of GALT [115]. Several DEG upregulated with ethanol consumption in the ileum are important for lymphocyte function and trafficking such as *CD19*, *CD27*, *CD37*, *CD40*, *CD44*, *CCL19*, *CCR6*, and *CCR7*. These changes in gene expression occurred despite the lack of differences in T cell frequencies [56] which suggests that ethanol regulates gene expression at the cell level rather than through changes in cell numbers. Moreover, we detected large increases in the expression of Toll-like receptors (TLR) that are critical in the detection of bacterial lipopeptides (*TLR1*), single-stranded RNA viruses (*TLR7*), and other unknown pathogen-associated molecular patterns (*TLR10*) [336]. STEM analysis also revealed increased levels of several genes associated with host defense and inflammation such as *CCL25*, *ACE1*, *DGAT1*, and *CD13*.

Interestingly, we found several DEG implicated in the initiation and/or progression of CRC in the ileum including the transcription factor lymphoid enhancer factor-1 (*LEF1*),

which is highly expressed in human CRC cells [337]. In addition, expression of *NOS2*, which plays a critical role in preventing tissue damage due to reactive oxygen species, was downregulated 6-fold with heavy drinking in the colon. Given that alcohol consumption increases production of reactive oxygen species [338], loss of *NOS2* expression can potentially exacerbate DNA damage and carcinogenesis. STEM also uncovered a cluster of 38 genes involved in "colonic diseases" that remain unchanged with non-heavy ethanol consumption but greatly increase with heavy ethanol consumption and included the metastasis suppressor gene *CD82*, and tumor protein translationally controlled-1 (*TPT1*), which protein levels have been shown to be drastically increased in the early stages of CRC development [339].

We also report an increase in the expression of gap junction protein *GJA5* in the ileum, which regulates the passage of ions and molecules between cells. Increased expression of *GJA5* could lead to an increase in translocation of bacteria, their products, and dietary antigens, which in turn leads to increased inflammation and could explain the robust upregulation of immune genes in the ileum. This change is in line with the increased endotoxin-core IgM antibody titers observed, and is potentially indicative of decreased barrier function. Indeed, a clinical study showed that a one-time consumption of 20g ethanol (non-heavy ethanol consumption) is sufficient to cause an increase in both small and large intestinal permeability in healthy human volunteers [130].

Fewer immune genes were dysregulated by ethanol consumption in the colon compared to the ileum, potentially due to reduced GALT in this compartment [115]. Moreover, in contrast to the ileum, the expression of genes involved in immune function

and regulation of inflammation were reduced in the colon following ethanol consumption including *PI3*, *IFIT3*, *IDO1*, *CCL19*, *CCR7*, and *DEF6*. The difference in direction of change could be due to the fact that immune cells in the colon have evolutionarily adapted to a more regulatory role due to the greater microbial load [340]. Reduced expression of immune genes could also signal reduced immune surveillance in the colon with long-term ethanol consumption, which in turn could facilitate the development of malignancies. For instance, expression of *IL21R*, which plays an important role in anti-tumor responses [341], is reduced in the colon. Other genes that negatively correlated with alcohol dose are responsible for control of the cell cycle including *CDC42*, *RAB1B*, and *RAB8A*.

Similarly, STEM analysis identified 2 distinct clusters of genes the expression of which negatively correlated with ethanol consumption in the colon. These genes enriched to the disease term "gastrointestinal neoplasms" including *CFL1*, *FABP*, *FTH1*, and *RACK1*. STEM also revealed that expression of miR-663, which is known to be dysregulated in several types of cancer [342], decreased with ethanol consumption. Decreased expression of this microRNA was primarily driven by non-heavy ethanol consumption in the colon, and was only slightly further exacerbated following heavy ethanol consumption.

Interestingly, we did not detect a difference in microbial community diversity in ethanol-consuming animals compared to controls at the group level. This observation is in contrast to a recent study using a rodent model of acute ethanol liquid diet, which reported a decrease in bacterial diversity with alcohol exposure [121]. The discrepancy between our data and the rodent studies could be due to differences in acute versus chronic consumption

of ethanol, the total amount of ethanol consumed, the diet (our macaques have concurrent access to 4% ethanol as well as water versus the liquid chow diet for the rodent study), the variable dose of ethanol consumed by our animals (since it is a voluntary ethanol consumption model) and last but not least physiological and genetics differences between rodents and nonhuman primates.

Furthermore, previous studies showed decreased abundance of *Bacteroidetes* and *Firmicutes* and higher abundance of *Proteobacteria* and *Actinobacteria* in the sigmoid colon and feces of AUD patients with ALD compared to those without ALD [154, 155]. In contrast, data reported here show minor differences at the phyla level that reflected a slight increase in *Bacteriodetes* and a reduction of *Proteobacteria*. This difference is likely due to the lack of ALD in our animals and examination of mucosa-associated bacterial communities, which can greatly differ from stool communities [343]. AUD has also been shown to result in an increase in the prevalence of families containing pathogenic bacteria including *Enterobacteriaceae*, *Streptococcaceae*, and *Prevotellaceae* as well as a reduction in beneficial families such as *Lachnospiraceae* in fecal material from patients with cirrhosis [155]. Similarly, we report that chronic ethanol consumption was positively associated with *Streptococcaceae*, *Prevotellaceae*, as well as *Lachnospiraceae* in the duodenum, ileum, and colon.

In the duodenum, we uncovered a positive correlation between ethanol consumption and several OTU associated with inflammation such as *Porphyromonadaceae* [344]. We also identified many OTU associated with inflammation that positively correlated with ethanol consumption in the ileum including major *Prevotella*

species (*P. copri*, *P. stercorea*, and *P. loescheii*). Similarly, the frequency of opportunistic pathogens such as *Prevotella copri*, *P. stercorea*, *P. loescheii, Clostridium perfrigens, C. celatum, C. clostridioforme*, and *Streptococcus suis* were increased with daily ethanol dose in the colon. In contrast, chronic heavy ethanol consumption decreased putatively beneficial bacteria such as *Blautia* species in jejunum and *Ruminococcus* species in ileum. Interestingly, an expansion of *P. copri* in mice has been shown to exacerbate colitis [345]. We also observed an increase in the genus *Porphyromonas* in the colon with heavy ethanol drinking compared to controls. *Porphyromonas* have been identified as a strong biomarker for CRC in humans [346]. Decreases in *Porphyromonadaceae* have also been associated with inflammation in colorectal tumor-bearing mice [344]. In the colon, we also found a positive correlation between ethanol consumption and frequency of *Peptostreptococcaceae bacterium* and *Haemophilus parainfluenzae*, which are enriched in CRC patients [332] and associated with colorectal carcinoma-in-adenoma [334]. Furthermore, *Selenomonas dianae*, which is enriched in proximal colon tumors [333], was also increased with ethanol consumption. These findings are particularly interesting given the increased incidence of CRC with AUD [15]. Additionally, the abundance of *Flexispira* species, which are usually dominant in healthy macaques [347], was reduced with heavy ethanol consumption suggestive of a loss in commensal bacteria. Furthermore, PICRUSt analyses revealed several putative bacterial genes that positively correlated with ethanol consumption are involved in carbohydrate metabolism. We also uncovered a negative correlation between ethanol consumption and the putative bacterial gene 4'-phosphopantetheinyl transferase,

which is involved in vitamin production and metabolism, a potential mechanism of alcohol-mediated malabsorption/malnutrition.

In summary, the studies presented in this manuscript show that ethanol consumption results in robust changes in gene expression, notably within the ileum where a large number of genes involved in host defense and inflammation were upregulated. Chronic ethanol consumption also leads to dysregulated expression of genes involved in GI cancers, especially CRC. Moreover, relative abundance of several bacterial taxa was altered throughout the GI tract including a dose-dependent decrease in the incidence of putatively beneficial bacteria as well as increased frequencies of taxa associated with inflammation and CRC. Similarly, *in silico* analysis suggests that several bacterial metabolic pathways may also be altered with ethanol consumption. It is important to note that animals used in our study lack liver damage, therefore the changes in gene expression and bacterial communities described precede overt clinical disease and could therefore potentially serve as prognostic biomarkers.

Limitations of our study include the use of full thickness biopsies, which precluded us from being able to trace changes in gene expression to specific cell types. Performing RNA-Seq on isolated cell populations (intestinal epithelial cells, lamina propria lymphocytes, intra-epithelial lymphocytes, and smooth muscle cells) may reveal additional gene expression changes within specific cell population that were diluted by the use of a full thickness biopsy. Moreover, the lack of access to biopsies before the animals were exposed to ethanol impeded our ability to conduct longitudinal studies. Access to fecal samples or luminal contents would have also allowed us to compare mucosa-associated

versus luminal communities and analyze microbial metabolites. Finally, we evaluated a small number of animals and only used male macaques. However, our study also has unique strengths, notably the use of an outbred nonhuman primate model, the analysis of biopsies from the 4 major gut sections, and the simultaneous measurement of changes in gene expression profiles and abundance of microbial communities with the same biopsy.

Future studies need to analyze bacterial communities using shotgun metagenomics and metabolomics. Additionally, the mechanisms underlying changes in gene expression will be explored by defining epigenetic changes such as histone modifications and alterations in microRNA profiles. The functional consequences of alterations in specific bacterial species will be determined by assessing their impact on barrier function, their ability to translocate, and invade and adhere to epithelial cells. Last but not least, these studies need to be extended to study female macaques in order to identify gender differences in the impact of ethanol consumption on GI health.

Chapter 4: Transcriptional profiling alters intestinal lamina propria lymphocyte function following chronic ethanol consumption

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Abstract

Chronic heavy alcohol consumption, also referred to as alcohol use disorder (AUD), results in intestinal injury characterized by increased permeability, dysbiosis, nutrient malabsorption, and increased risk of colorectal cancer. Additionally, studies have reported a reduction in frequency that is often accompanied by an increase in proliferation of mucosal immune cells. Our understanding of the mechanisms by which ethanol consumption results in intestinal injury remains incomplete. Previous studies from our laboratory showed large immune gene expression changes in ileum and colonic biopsies obtained from male macaques following 12 months of voluntary ethanol consumption. Therefore, in this study we investigated ethanol-mediated changes in transcriptional and functional responses of lamina propria lymphocytes (LPL) isolated from the same animals and four major sections of the gut as our recent publication. In response to PMA/ionomycin, ethanol-consuming animals displayed larger transcriptional changes than controls, several of which were downregulated genes involved in metabolic processes. Furthermore, increased expression of inflammatory and defense response genes in response to stimulation was detected in animals that consumed moderate amounts of ethanol. Cytokine, chemokine, and growth factor production was altered in animals that consumed ethanol and suggest aberrant inflammatory responses. The altered intestinal LPL function detected in our study reveals novel insight into the contribution of intestinal injury associated with excess alcohol consumption.

Introduction

The intestinal mucosal immune system acts as the first line of defense against enteropathogens. It is crucial that this compartment maintain tight regulation due to its complex job of distinguishing harmful antigens from natural gut microbiota. Intestinal lamina propria lymphocytes (LPL), which are predominately CD4+ T cells, are antigen-experienced effector lymphocytes distributed along the gastrointestinal tract [348]. In contrast to the peripheral immune system, the mucosal immune system of the gut contains a large number of activated effector lymphocytes even in the absence of disease or infection due to increased antigen exposure.

Excessive alcohol consumption, also referred to as alcohol use disorder (AUD), leads to increased susceptibility to infection, severe intestinal damage, compromised mucosal barrier, and overall loss of gut homeostasis. Duodenal biopsies obtained from patients with AUD showed an increase in the number of B-lymphocytes in the lamina propria, accompanied by a decrease in the number of intraepithelial T-lymphocytes, and macrophages in comparison to biopsies from healthy controls [137]. A more recent study showed CD161+ mucosa-associated invariant T cells were reduced in numbers, hyperactivated (increased expression of CD69), and displayed defective antibacterial cytokine and cytotoxic responses from AUD patients with increased intestinal permeability and liver disease compared to healthy controls [141]. A rhesus macaque chronic binge alcohol administration via intra-gastric catheter model found a decrease in absolute numbers of jejunal lamina propria T-lymphocytes, accompanied by increases in T-lymphocyte proliferation and turnover following 3 months of chronic alcohol consumption

[142]. Ethanol-fed mice showed a marked loss of both B and T lymphocytes from gut-associated lymphoid tissue [138] and increased susceptibility to the intestinal pathogens *Listeria monocytogenes* and *Salmonella typhimurium* but not *Nippostrongylus brasiliensis* suggestive of a defect in Th1 immunity [139]. A rat model of acute alcohol and burn injury showed a significant suppression in IL-2 and IFN-γ production, and T cell proliferation from gut-associated lymphoid tissue [143]. A study utilizing ethanol-fed mice found, in addition to a reduction in T-lymphocyte numbers, an increase in apoptosis in the jejunal and colonic lamina propria compared to control animals [144].

Our understanding of the mechanisms involved in alcohol-mediated disruptions of intestinal LPL function remains incomplete. In addition, modulation of LPL function by moderate ethanol consumption has been largely understudied. Finally, because of the regional specialization and compartmentalization of the intestinal immune system, it is imperative that we elucidate the impact of ethanol on LPL function in each major section of the gut. To address these questions, we leverage a nonhuman primate model of voluntary ethanol self-administration [33, 34] to investigate the dose-dependent and site-specific impact of alcohol consumption on LPL response to stimulation. Specifically, we compared the transcriptional profiles of LPLs isolated from the duodenum, jejunum, ileum, and colon of male ethanol-consuming and control macaques before and after PMA/ionomycin.

Materials and Methods

Rhesus macaque model of voluntary ethanol self-administration

We leveraged the Monkey Alcohol Tissue Research Resource (MATRR; http://www.matrr.com/) to conduct the experiments described in this manuscript. Samples from 12 male rhesus macaques aged 4-5 years of age were used in these studies (Table 4.1). Eight of these male rhesus macaques chronically consumed 4% ethanol in water for 12 months and segregated into two cohorts, n=4 each, based on average blood ethanol (BEC) values: non-heavy drinkers with an average BEC of 22.3-48.8 mg/dl (or mean daily intake of 1.8-2.3 g/kg) and heavy drinkers with an average BEC of 90-126 mg/dl (or mean daily intake of 2.8-3.3 g/kg) as previously described in [169]. Four males served as controls and consumed a calorically-matched maltose dextrose solution.

Although controls and ethanol-consuming animals consumed comparable numbers of total calories, heavy drinkers consumed a much higher number of ethanol-derived calories compared to non-heavy drinkers [349]. All 12 animals were euthanized after 12 months of open access and biopsies were collected from duodenum, jejunum, ileum, and traverse colon. Ethanol-consuming animals did not show overt signs of liver damage as assessed by measuring alanine transaminase (ALT) and aspartate transaminase (AST) enzyme levels and histological assessment of liver sections [349].

Animal ID	Mean daily ethanol intake (g/kg/day)	Blood Ethanol Content (Avg mg%)	Drinking status	Duodenum LPLs		Jejunum LPLs		Ileum LPLs		Colon LPLs	
				NS	S	NS	S	NS	S	NS	S
C1	0	0	Control	X	X	X	X	X	X		
C2	0	0	Control	X	X	X	X	X	X	X	X
C3	0	0	Control	X	X	X	X	X	X	X	X
M1	1.8	35.86	Moderate	X	X	X	X	X	X		
M2	1.9	32.68	Moderate	X	X	X	X	X	X		
M3	1.9	20.74	Moderate	X	X	X	X	X	X		
M4	2.3	33.89	Moderate			X	X				
H1	2.8	67.42	Heavy	X	X	X	X	X	X	X	X
H2	3	75.02	Heavy	X	X	X	X	X	X		
H3	3.1	72.24	Heavy	X	X	X	X	X	X	X	X

Table 4.1: Characteristics of macaques used in these studies. (NS=No Stimulation; S=PMA/ionomycin Stimulation). Boxes shaded grey represent RNA-Seq libraries of poor quality and were thus excluded from the RNA-Seq analysis.

Lamina propria lymphocyte (LPL) collection and stimulation

Tissues were collected following terminal anesthesia (8 mg/kg ketamine followed by intravenous pentobarbital). Duodenum, jejunum, ileum, and descending colon were collected in RPMI supplemented with 3% fetal bovine serum, streptomycin/penicillin, and L-glutaine. LPLs from were isolated using DTT/EDTA and collagenase IX/DNAse digest and then purified over Percoll gradient (GE Healthcare Biosceinces, Pittsburgh, PA). Cells were cryopreserved in Fetalplex Animal Serum Complex (Gemini Bio-Products, West Sacramento, CA) and DMSO. $5x10^5$ cells were stimulated for 12 hours at 37°C in 5% CO_2 in supplemented RPMI only or with phorbol myristate acetate (PMA)/ionomycin or RP10 for 12 hours.

RNA sequencing (RNA-Seq)

Total RNA was isolated from duodenal, jejunal, ileal, and colonic lamina propria lymphocytes using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany). RNA concentration and quality was verified using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Libraries were constructed using the TruSeq Stranded RNA LT Ribo-Zero H/M/R kit (Illumina, San Diego, CA, USA). Briefly, rRNA-depleted RNA was fragmented and converted to double stranded cDNA. Adapters were ligated and ~300 base pair fragments were amplified by PCR and selected by size exclusion. Each library was labeled with a unique barcode for multiplexing. To ensure proper sizing, quantitation, and quality prior to sequencing, libraries were analyzed on the Agilent 2100 Bioanalyzer. Multiplexed libraries were subjected to single-end 86 or 100 base pair sequencing using

the Illumina NextSeq 500 (jejunal and ileal LPL) or HiSeq 4000 (duodenal and colonic LPL) platforms, respectively.

RNA-Seq bioinformatic analysis

Data analysis was performed with the RNA-Seq workflow module of the *systemPiperR* package available on Bioconductor [170, 171]. Quality reports were generated with *seeFastq*. Reads were mapped with the splice-aware aligner suite Bowtie2/Tophat2 [172, 173] against the *Macaca mulatta* genome from Ensembl [174]. Default parameters of Tophat2 optimized for mammalian genomes were used for alignment (allowing 2% nucleotide mismatches). Raw expression values in form of gene-level read counts were generated with *summarizeOverlaps* [175]. Only reads overlapping exonic regions of genes were counted, discarding reads mapping to ambiguous regions of exons from overlapping genes.

Analysis of differentially expressed genes (DEG) was performed with the generalized linear model likelihood ratio test method from the *edgeR* package [176, 177]. To determine the impact of ethanol consumption on gene expression, we carried out three different comparisons: (1) resting LPLs isolated from controls versus resting LPLs isolated from ethanol-consuming animals; (2) resting LPLs isolated from controls versus stimulated LPLs isolated from controls; and (3) resting LPLs isolated from ethanol-consuming animals versus stimulated LPLs isolated from ethanol-consuming animals. DEG were defined as those with a fold change of ≥ 2 and a Benjamini-Hochberg-controlled false discovery rate (FDR) of <0.05. Only characterized protein coding genes with human

homologs and a median value of ≥5 reads per kilobase of transcript per million mapped reads (RPKM) in each group were included for further analysis. Functional enrichment analysis was performed to identify significant biological pathways including gene ontology (GO) terms and disease biomarkers using MetaCore™ software (GeneGo). The software program Short Time-series Expression Miner (STEM; v1.3.11) was utilized to further identify clusters of genes that show similar patterns of expression with alcohol dose: none, non-heavy, and heavy [321]. Heatmaps for gene expression represent the absolute normalized expression (RPKM); range of colors is based on scaled and centered RPKM values of the entire set of genes (red represents increased expression while blue represents decreased expression).

Luminex

LPL culture supernatant samples were analyzed using Cytokine Monkey Magnetic 29-Plex Panel (Life Technologies, Grand Island, NY) to measure protein levels of 13 cytokines (IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-12, IL-15, IL-17, TNFα, IL-1RA, IL-10, and MIF), 6 growth factors (EGF, FGF-basic, G-CSF, GM-CSF, HGF, and VEGF), and 10 chemokines (MCP-1, MIP-1α, MIP-1β, RANTES, EOTAXIN, MDC, IL-8, MIG, IP-10, and I-TAC) per the manufacturer's instructions. Samples were run in duplicates.

Statistical analysis

Statistical significance of protein values between controls and ethanol-consuming animals was assessed with unpaired t-test followed by Bonferroni's multiple comparison correction tests. These analyses were carried out by GraphPad Prism version 6 (GraphPad software).

Results

Chronic ethanol consumption modulates gene expression in resting LPLs in a dose-dependent manner

To examine the impact of chronic alcohol consumption on mucosal immune function, we compared the transcriptional profiles of resting LPLs isolated from the duodenum, jejunum, ileum, and colon of male ethanol-consuming and control macaques. Flow cytometric analysis revealed that approximately 75% of the isolated LPLs were T cells (Fig. 4.1). We did not uncover any differentially expressed genes (DEGs) in resting duodenal, jejunal, and colonic LPLs obtained from controls and ethanol-consuming animals. In contrast, 35 DEGs (4 upregulated and 31 downregulated) were detected in ileal LPLs (Fig. 4.2A). The 4 genes with increased expression levels include mitogen-activated protein kinase kinase kinase (*MAP3K12*), cysteinyl-tRNA synthetase-2 (*CARS2*), coronin, actin binding protein-1B (*CORO1B*), and shisa family member-5 (*SHISA5*). The 31 genes with decreased expression levels functionally enriched to Gene Ontology (GO) terms "regulation of localization" (FDR-P=0.00245; 17 genes), "calcium-mediated signaling" (FDR-P=0.00104; 5 genes), and "cytoskeleton organization" (FDR-P=0.00301; 10 genes).

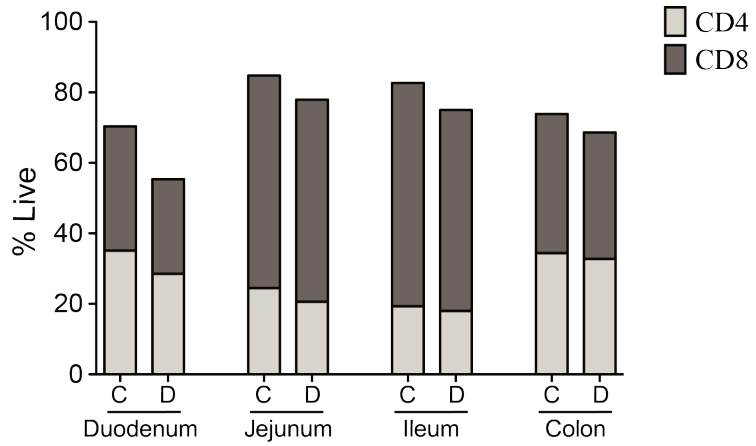
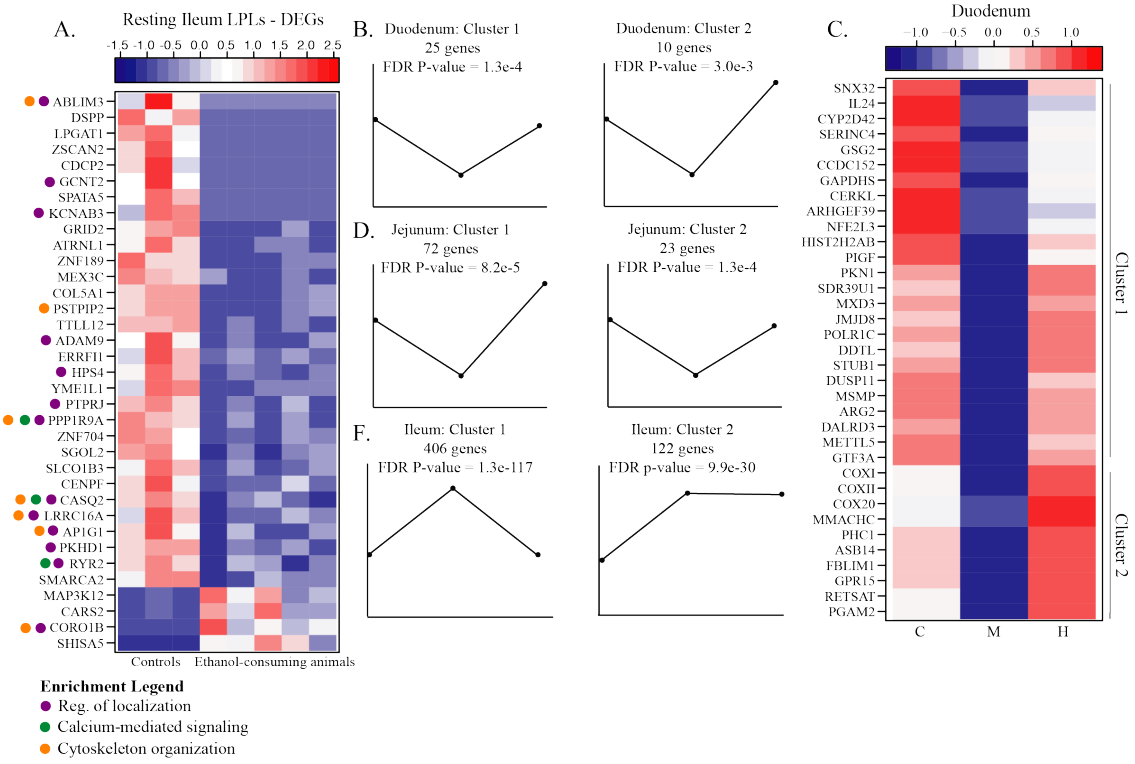
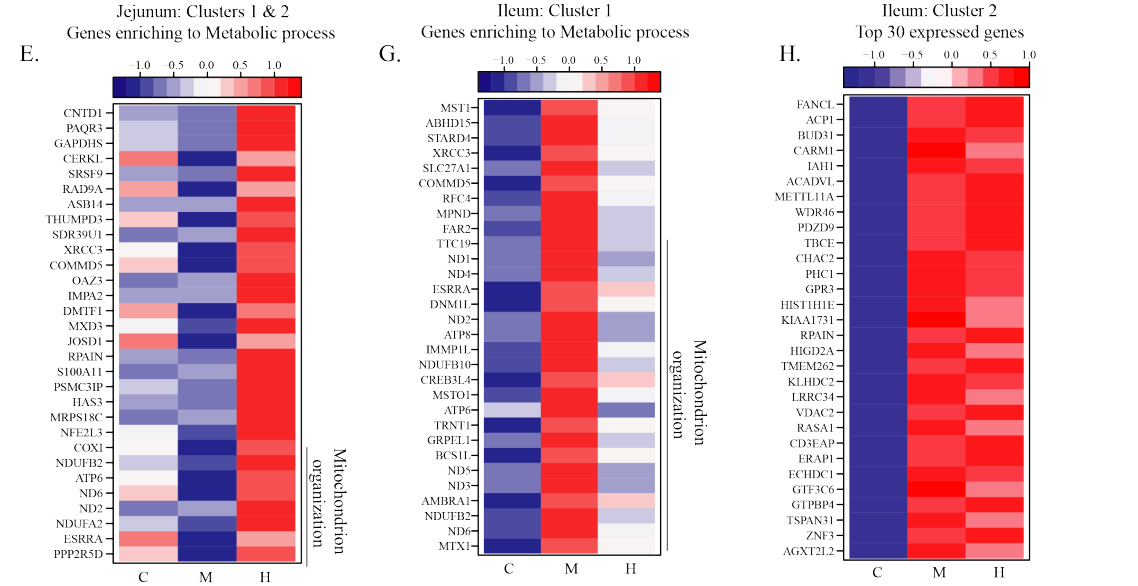


Figure 4.1: Flow cytometric analysis reveals LPLs are primarily CD4+ and CD8+ T cells. Bar graphs displaying the frequency of CD4+ and CD8+ T cells in controls (C) and drinkers (D) in each gut section as determined by flow cytometry.

Since we collected LPLs from male macaques with varying ethanol intake, we next investigated dose-dependent dynamic changes in gene expression using the open source software Short Time-series Expression Miner (STEM). Animals were divided into 3 groups based on their ethanol consumption patterns: controls (n=3), non-heavy drinkers (n=4), and heavy drinkers (n=3). This analysis could not be carried out for colonic LPLs because we did not have access to cells from non-heavy drinkers. In resting duodenal LPLs, STEM analysis identified 2 clusters of genes the expression of which decreases with non-heavy ethanol consumption then increases to either control or above control levels with heavy drinking (Fig. 4.2B). Genes in cluster 1 play a role in the regulation of gene transcription including *POLR1C* and *GTF3A*, and chromatin organization such as *HIST2H2AB*. Genes in cluster 2 play a role in respiration and functionally enriched to GO terms such as "oxidation-reduction process" (FDR-P=0.01578; 5 genes; e.g. *MMACHC*, *PGAM2*; Fig. 4.2C). Similarly, in resting jejunum LPLs, STEM analysis identified two clusters of genes the expression of which decreases with non-heavy ethanol consumption then increases to either control or above control levels with heavy drinking (Fig. 4.2D). Jejunal genes in these clusters enriched to "metabolic process" (FDR-P=0.01889; 62 genes) "mitochondrion organization" (FDR-P=0.001439; 12 genes; Fig. 4.2E). In contrast, STEM analysis identified 2 clusters of genes the expression of which increases with non-heavy ethanol consumption then decreases to either control levels or remains unchanged with heavy drinking in resting ileal LPLs (Fig. 4.2F). As described for the jejunum, the ileal genes in clusters 1 and 2 functionally enriched to the GO terms "metabolic process" (FDR-P=7.058e-7; 298 genes; e.g. *SLC27A1*, *FAR2*; Fig. 4.2G; and FDR-P=0.0161; 95 genes;

e.g. *ERAP1*, *MLX*; Fig. 4.2H) and “mitochondrion organization” (FDR-P=0.005257; 26 genes; e.g. *ND1-6*; Fig. 4.2G; and FDR-P=0.02916; 8 genes; e.g. *WDR46*, *PLA2G10*; Fig. 4.2H).

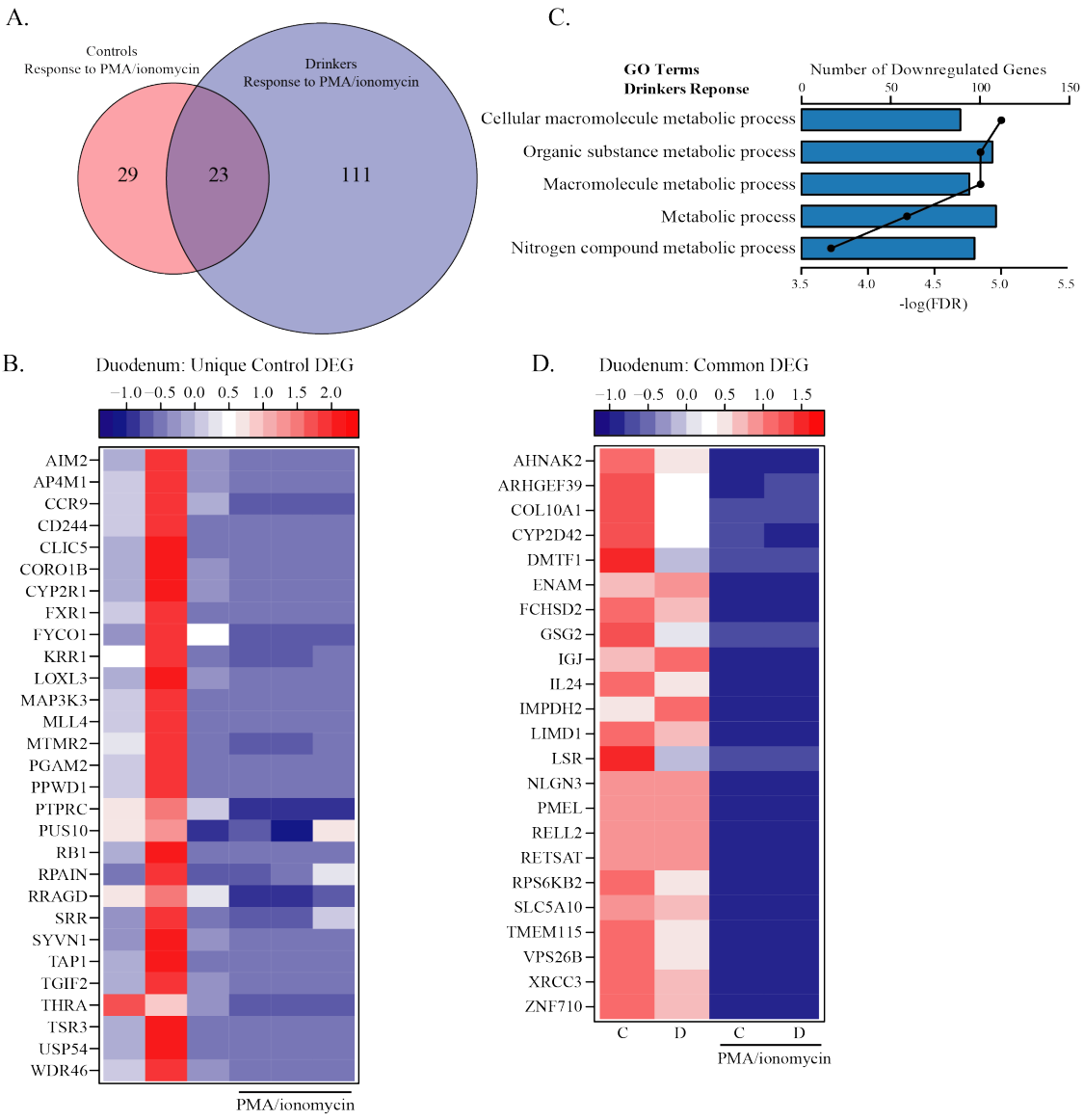

A.
Resting Ileum LPLs - DEGs
-1.5 -1.0 -0.5 0.0 0.5 1.0 1.5 2.0 2.5
ABLIM3
DSPP
LPGAT1
ZSCAN2
CDCP2
GCNT2
SPATA5
KCNAB3
GRID2
ATRNL1
ZNF189
MEX3C
COL5A1
PSTPIP2
TTLL12
ADAM9
ERRFI1
HPS4
YME1L1
PTPRJ
PPP1R9A
ZNF704
SGOL2
SLCO1B3
CENPF
CASQ2
LRRC16A
AP1G1
PKHD1
RYR2
SMARCA2
MAP3K12
CARS2
CORO1B
SHISA5
Controls
Ethanol-consuming animals
B.
Duodenum: Cluster 1
25 genes
FDR P-value = 1.3e-4
Duodenum: Cluster 2
10 genes
FDR P-value = 3.0e-3
D.
Jejunum: Cluster 1
72 genes
FDR P-value = 8.2e-5
Jejunum: Cluster 2
23 genes
FDR P-value = 1.3e-4
F.
Ileum: Cluster 1
406 genes
FDR P-value = 1.3e-117
Ileum: Cluster 2
122 genes
FDR p-value = 9.9e-30
C.
Duodenum
−1.0 −0.5 0.0 0.5 1.0
SNX32
IL24
CYP2D42
SERINC4
GSG2
CCDC152
GAPDHS
CERKL
ARHGEF39
NFE2L3
HIST2H2AB
PIGF
PKN1
SDR39U1
MXD3
JMJD8
POLR1C
DDTL
STUB1
DUSP11
MSMP
ARG2
DALRD3
METTL5
GTF3A
COX1
COXII
COX20
MMACHC
PHC1
ASB14
FBLIM1
GPR15
RETSAT
PGAM2
C
M
H
Cluster 1
Cluster 2
Enrichment Legend
Reg. of localization
Calcium-mediated signaling
Cytoskeleton organization


Jejunum: Clusters 1 & 2
Genes enriching to Metabolic process
E.
−1.0 −0.5 0.0 0.5 1.0
CNTD1
PAQR3
GAPDHS
CERKL
SRSF9
RAD9A
ASB14
THUMPD3
SDR39U1
XRCC3
COMMD5
OAZ3
IMPA2
DMTF1
MXD3
JOSD1
RPAIN
S100A11
PSMC3IP
HAS3
MRPS18C
NFE2L3
COX1
NDUFB2
ATP6
ND6
ND2
NDUFA2
ESRRA
PPP2R5D
C
M
H
Mitochondrion organization
Ileum: Cluster 1
Genes enriching to Metabolic process
G.
−1.0 −0.5 0.0 0.5 1.0
MST1
ABHD15
STARD4
XRCC3
SLC27A1
COMMD5
RFC4
MPND
FAR2
TTC19
ND1
ND4
ESRRA
DNM1L
ND2
ATP8
IMMP1L
NDUFB10
CREB3L4
MSTO1
ATP6
TRNT1
GRPEL1
BCS1L
ND5
ND3
AMBRA1
NDUFB2
ND6
MTX1
C
M
H
Mitochondrion organization
Ileum: Cluster 2
Top 30 expressed genes
H.
−1.0 −0.5 0.0 0.5 1.0
FANCL
ACP1
BUD31
CARM1
IAH1
ACADVL
METTL11A
WDR46
PDZD9
TBCE
CHAC2
PHC1
GPR3
HIST1H1E
KIAA1731
RPAIN
HIGD2A
TMEM262
KLHDC2
LRRC34
VDAC2
RASA1
CD3EAP
ERAP1
ECHDC1
GTF3C6
GTPBP4
TSPAN31
ZNF3
AGXT2L2
C
M
H

(Previous page) Figure 4.2: Chronic ethanol consumption modulates gene expression in resting LPLs in a dose-dependent manner. (A) Heatmap depicting expression levels of the differentially expressed genes (DEGs) that have corresponding characterized human homologs in resting ileal LPLs showing individual controls and ethanol-consuming animals. Colored dots represent characterized human homologs that enrich to the listed Gene Ontology (GO) terms in the Enrichment Legend. (B) Line graphs depicting the dose-dependent dynamics changes in gene expression in resting duodenal LPLs. (C) Heatmap of characterized human homologs in duodenum STEM clusters 1 and 2 showing median expression levels of resting duodenal LPLs from controls, moderate drinkers, and heavy drinkers. (D) Line graphs depicting the dose-dependent dynamics changes in gene expression in resting jejunal LPLs. (E) Heatmap of the top 30 characterized human homologs enriching to the Gene Ontology (GO) term "metabolic process" (n=60) including genes that also enrich to the GO term "mitochondrion organization" (n=8) in jejunum STEM clusters 1 and 2 showing median expression levels of resting jejunal LPLs from controls, moderate drinkers, and heavy drinkers. (F) Line graphs depicting the dose-dependent dynamics changes in gene expression in resting ileal LPLs. (G) Heatmap of the top 30 characterized human homologs enriching to the Gene Ontology (GO) term "metabolic process" (n=298) including genes that also enrich to the GO term "mitochondrion organization" (n=22) in ileum STEM cluster 1 showing median expression levels of resting ileal LPLs from controls, moderate drinkers, and heavy drinkers. (H) Heatmap of the top 30 highly expressed characterized human homologs in ileum STEM cluster 2 showing median expression levels of resting ileal LPLs from controls, moderate drinkers, and heavy drinkers.

Chronic ethanol consumption modulates the expression of genes associated with metabolic processes in duodenal LPLs following PMA/ionomycin stimulation

We next compared the transcriptional profiles of PMA/ionomycin stimulated duodenal LPLs, which showed 52 DEGs (3 upregulated and 49 downregulated) in control animals versus 134 in ethanol-consuming animals (0 upregulated and 134 downregulated) (Fig. 4.3A). Of the 52 DEG detected in controls, 4 play a role in T cell function (*CCR9*, *CD244*, *PTPRC, TAP1*; Fig. 4.3B). Of the 134 DEG in drinkers, the majority functionally enriched to GO terms associated with metabolism such as "cellular macromolecule metabolic process" (FDR-P=9.923e-6; 89 genes; e.g. *BRD7*, *CREB3L4*), "organic substance metabolic process" (FDR-P=1.421e-5; 107 genes; e.g. *DERL3*, *CD244*, *PTPRC*; Fig. 4.3C), and "metabolic process" (FDR-P=5.084e-5; 109 genes; e.g. *DERL3*, *CD244*, *PTPRC*). The 23 DEGs detected in both groups after stimulation are associated with cell differentiation including the transcription factor *DMTF1* and the scaffold protein *LIMD1* (Fig. 4.3D).


A.
Controls
Response to PMA/ionomycin
Drinkers
Response to PMA/ionomycin
29
23
111
C.
GO Terms
Drinkers Reponse
Number of Downregulated Genes
0
50
100
150
Cellular macromolecule metabolic process
Organic substance metabolic process
Macromolecule metabolic process
Metabolic process
Nitrogen compound metabolic process
3.5
4.0
4.5
5.0
5.5
-log(FDR)
B.
Duodenum: Unique Control DEG
−1.0 −0.5 0.0 0.5 1.0 1.5 2.0
AIM2
AP4M1
CCR9
CD244
CLIC5
CORO1B
CYP2R1
FXR1
FYCO1
KRR1
LOXL3
MAP3K3
MLL4
MTMR2
PGAM2
PPWD1
PTPRC
PUS10
RB1
RPAIN
RRAGD
SRR
SYVN1
TAP1
TGIF2
THRA
TSR3
USP54
WDR46
PMA/ionomycin
D.
Duodenum: Common DEG
−1.0 −0.5 0.0 0.5 1.0 1.5
AHNAK2
ARHGEF39
COL10A1
CYP2D42
DMTF1
ENAM
FCHSD2
GSG2
IGJ
IL24
IMPDH2
LIMD1
LSR
NLGN3
PMEL
RELL2
RETSAT
RPS6KB2
SLC5A10
TMEM115
VPS26B
XRCC3
ZNF710
C
D
C
D
PMA/ionomycin

(Previous page) Figure 4.3: Chronic ethanol consumption modulates the expression of genes associated with metabolic processes in duodenal LPLs. (A) Venn diagram depicting the overlap of the total number of differentially expressed characterized human homologs between controls and drinkers in response to PMA/ionomycin. (B) Heatmap of the 29 unique characterized human homologs in controls showing expression levels in individual control animals before and after PMA/ionomycin. (C) Heatmap of the common 23 characterized human homologs differentially expressed among controls and drinkers showing median expression levels of controls and drinkers before and after PMA/ionomycin. (D) Bar graph displaying the number of characterized human homologs downregulated in controls response to PMA/ionomycin that enriched to the listed Gene Ontology (GO) terms. Line represents the -log(FDR) associated with each GO term.

Chronic ethanol consumption alters gene expression in LPLs isolated from the jejunum

PMA/ionomycin stimulation resulted in 18 DEGs (2 upregulated genes and 16 downregulated) in samples from control animals and 37 DEGs in the samples from ethanol-consuming animals (20 upregulated genes and 17 downregulated; Fig. 4.4A). Of the 11 DEGs detected exclusively in controls, collagen type VII alpha-1 (*COL7A1*) and anaphase promoting complex subunit 1 (*ANAPC1*) showed increased expression levels, while genes important for cellular response to IFN-β (*GBP3*) and chromatin silencing (*HIST2H2AB*, *SUV39H1*) decreased their expression levels (Fig. 4.4B). Of the 30 DEGs unique to drinkers, *HTR2B*, *MCOLN3*, *SLC24A5*, and *XLC1* enriched to the GO term "calcium ion transport" (FDR-P=3.693e-5; 4 genes; Fig. 4.4B). Common DEGs were associated with T cell proliferation including *CD244* and *IL24* (Fig. 4.4B).

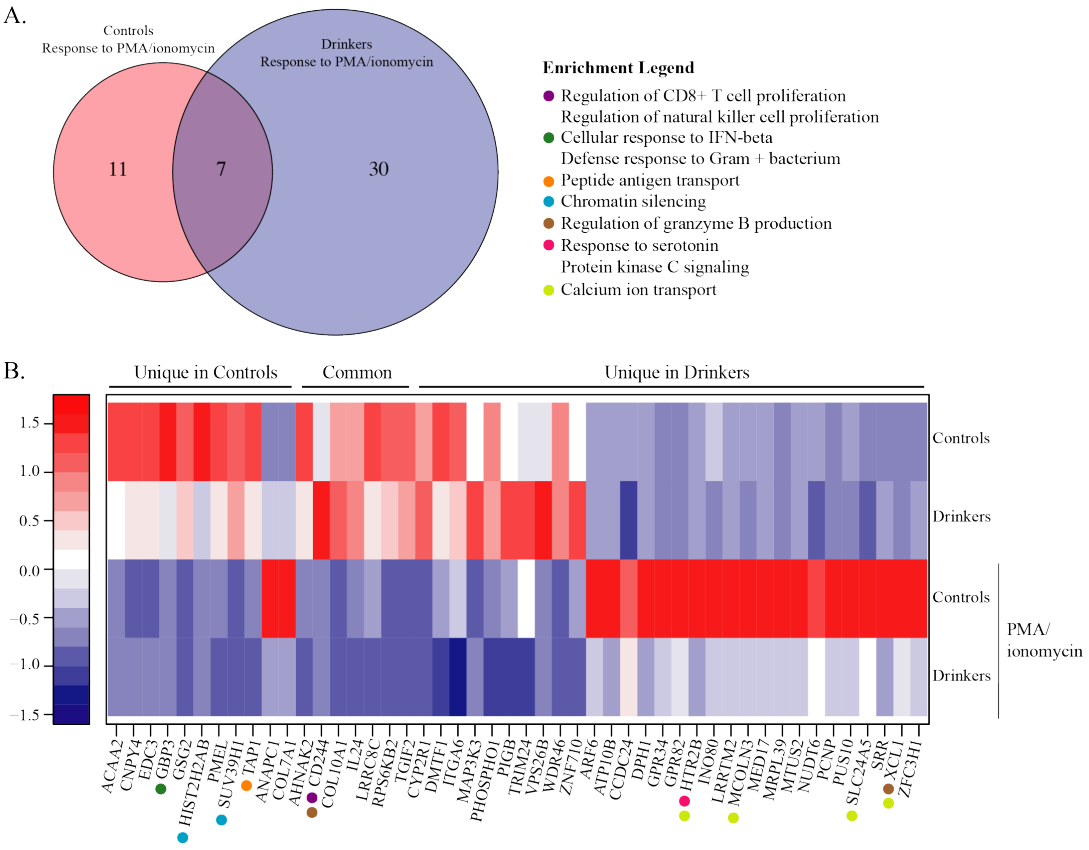
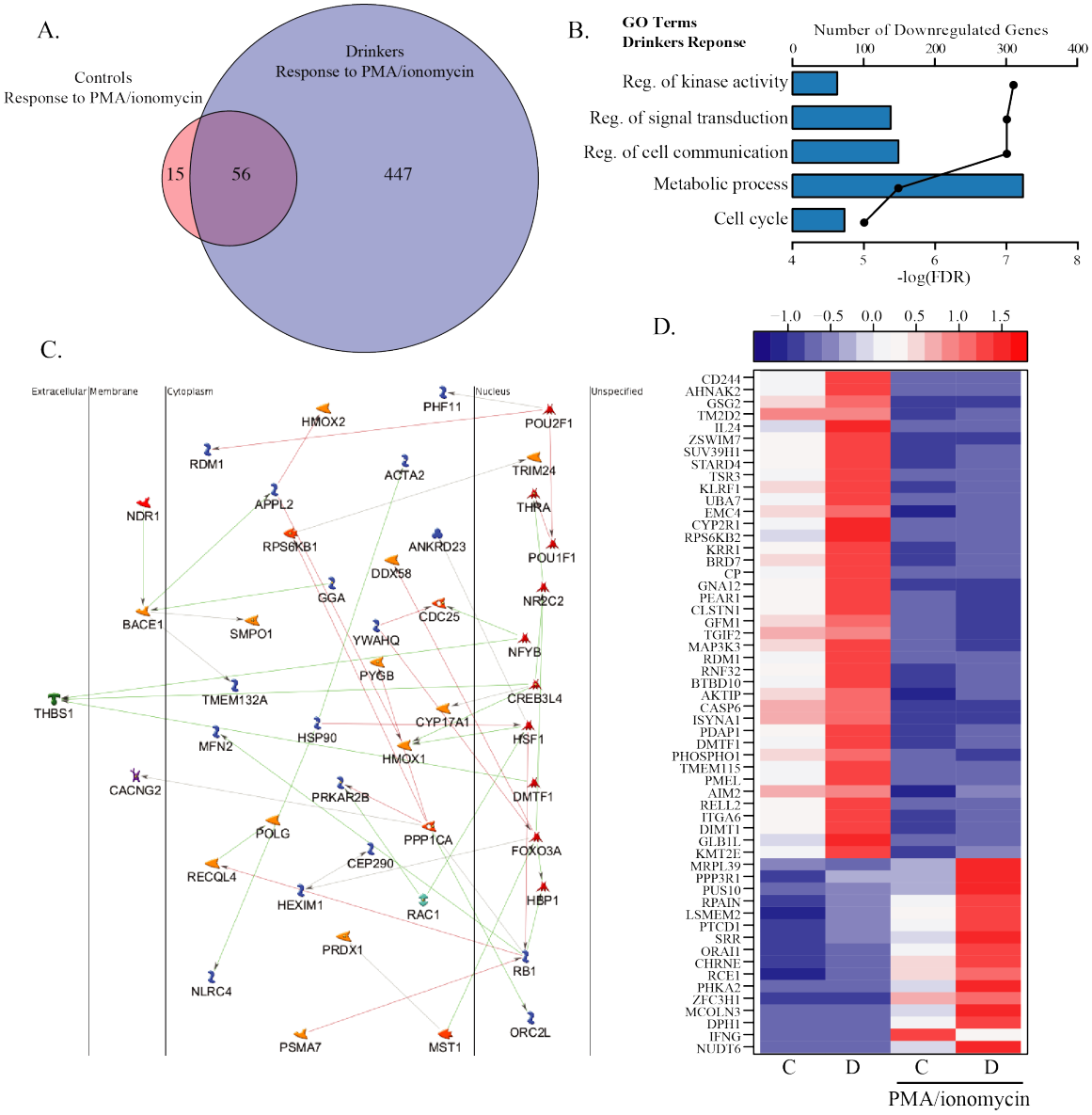


Figure 4.4: Chronic ethanol consumption alters gene expression in LPLs isolated from the jejunum. (A) Venn diagram depicting the overlap of the total number of characterized human homologs between controls and drinkers in response to PMA/ionomycin. (B) Heatmap of unique characterized human homologs in controls, common characterized human homologs among controls and drinkers, and unique characterized human homologs in drinkers showing median expression levels of controls and drinkers before and after PMA/ionomycin. Colored dots represent characterized human homologs that enrich to the listed Gene Ontology (GO) terms in the Enrichment Legend.

Aberrant expression of metabolic genes in ileal LPLs isolated from drinkers

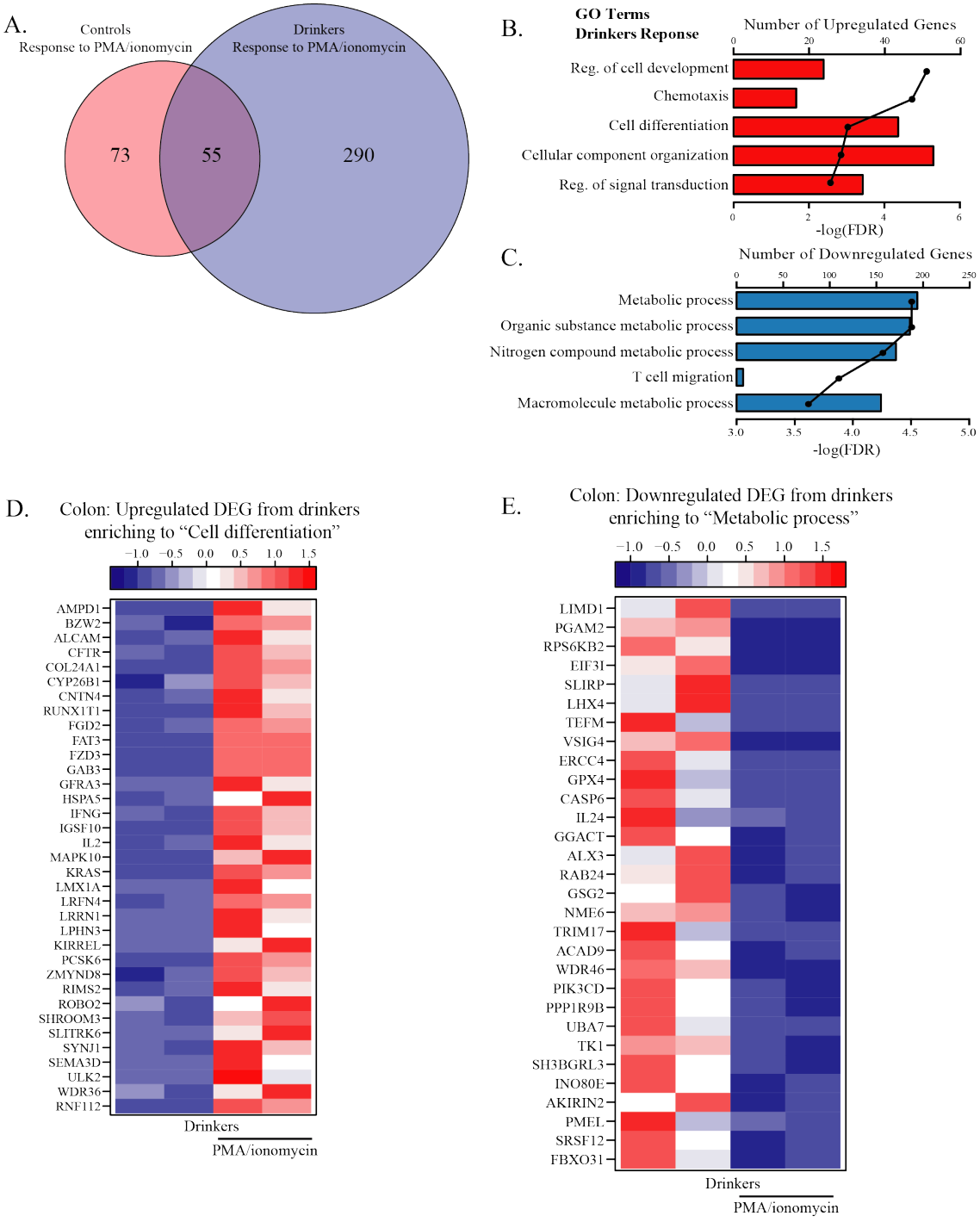
In ileal LPLs, 71 DEGs were identified in samples from controls (22 upregulated and 49 downregulated) and 503 DEGs in samples from ethanol-consuming animals (72 upregulated and 431 downregulated) (Fig. 4.5A). DEGs detected in control samples play a role in signaling (e.g. *PEAR1*, *RPS6KB2*) and T cell activation (e.g. *IFNG*, *NFKBIA*, *PPP3R1*). DEGs detected in PMA/ionomycin-stimulated ileal LPLs from ethanol-consuming animals enriched to GO terms related to cellular metabolism, signaling, and cell cycle (Fig. 4.5B). Of the DEGs that enriched to "metabolic process" (FDR-P=3.280e-6; 300 genes), 46 directly interact with one another (Fig. 4.5C). These interactions include genes important for the response to stress (*HSP90*, *HSF1*, *NLRC4*), cell cycle progression (*CDC25*, *HBP1*), and cell growth (*RAC1*, *ACTA2*). The 56 DEGs detected in LPLs from both controls and ethanol-consuming animals following stimulation play a role in immunity including *CD244*, *IL24*, and *KLRF1*; metabolism (*SRR*); proliferation (*NUDT6*); and apoptosis (*PUS10*) (Fig. 4.5D).


A.
Controls
Response to PMA/ionomycin
Drinkers
Response to PMA/ionomycin
15
56
447
B.
GO Terms
Drinkers Reponse
Number of Downregulated Genes
0
100
200
300
400
Reg. of kinase activity
Reg. of signal transduction
Reg. of cell communication
Metabolic process
Cell cycle
4
5
6
7
8
-log(FDR)
C.
Extracellular
Membrane
Cytoplasm
Nucleus
Unspecified
HMOX2
PHF11
POU2F1
RDM1
ACTA2
TRIM24
APPL2
THRA
NDR1
RPS6KB1
ANKRD23
POU1F1
DDX58
GGA
NR2C2
CDC25
BACE1
SMPO1
YWHAQ
NFYB
PYGB
TMEM132A
CREB3L4
THBS1
CYP17A1
HSP90
HSF1
MFN2
HMOX1
CACNG2
PRKAR2B
DMTF1
POLG
PPP1CA
FOXO3A
CEP290
RECQL4
HEXIM1
HBP1
RAC1
PRDX1
RB1
NLRC4
ORC2L
PSMA7
MST1
D.
−1.0
−0.5
0.0
0.5
1.0
1.5
CD244
AHNAK2
GSG2
TM2D2
IL24
ZSWIM7
SUV39H1
STARD4
TSR3
KLRF1
UBA7
EMC4
CYP2R1
RPS6KB2
KRR1
BRD7
CP
GNA12
PEAR1
CLSTN1
GFM1
TGIF2
MAP3K3
RDM1
RNF32
BTBD10
AKTIP
CASP6
ISYNA1
PDAP1
DMTF1
PHOSPHO1
TMEM115
PMEL
AIM2
RELL2
ITGA6
DIM1I1
GLB1L
KMT2E
MRPL39
PPP3R1
PUS10
RPAIN
LSMEM2
PTCD1
SRR
ORAI1
CHRNE
RCE1
PHKA2
ZFC3H1
MCOLN3
DPH1
IFNG
NUD16
C
D
C
D
PMA/ionomycin

(Previous page) Figure 4.5: Resting ileal LPLs isolated from drinkers are in a hyper-inflammatory state before response to stimulus. (A) Venn diagram depicting the overlap of the total number of differentially expressed characterized human homologs between controls and drinkers in response to PMA/ionomycin. (B) Bar graph displaying the number of characterized human homologs with reduced expression levels in drinkers response to PMA/ionomycin that enriched to the listed Gene Ontology (GO) terms. Line represents the -log(FDR) associated with each GO term. (C) Network image of the 46 direct interactions amongst the downregulated characterized human homologs that enriched to "metabolic process" (n=301). (D) Heatmap of the 56 common differentially expressed characterized human homologs between controls and drinkers showing median expression levels of controls and drinkers before and after PMA/ionomycin.

Expression of genes associated with cellular differentiation is altered by ethanol in colonic LPLs

We next compared the transcriptomes of colonic LPLs following PMA/ionomycin stimulation (n=2/group) (Fig. 4.6A). We detected 128 DEG in colonic LPLs from control animals (35 upregulated genes and 93 downregulated genes). Genes with increased expression levels enriched to GO terms such as "positive regulation of immune response" (FDR-P=0.03546; 9 genes; e.g. *IL2*, *IFNG*) and "cell differentiation" (FDR-P=0.0446; 18 genes; e.g. *DOCK1*, *NRG1*). The downregulated DEGs enriched to GO terms such as "cAMP-mediated signaling" (FDR-P=4.173e-4; 8 genes; e.g. *S1PR4*, *DGKQ*) and "wound healing" (FDR-P=0.005; 13 genes; e.g. *AHNAK2*, *NINJ1*). In ethanol-consuming animals, we identified 345 DEGs following stimulation: 87 upregulated genes and 258 downregulated genes (Fig. 4.6B and C). Several of the genes with increased expression enriched to GO terms such as "regulation of cell development" (FDR-P=7.596e-6; 23 genes; e.g. *CNTN4*, *FZD3*), "cell differentiation" (FDR-P=9.271e-4; 42 genes; e.g. *AMPD1*, *LPHN3*; Fig. 4.6D), and "regulation of signal transduction" (FDR-P=0.002681; 33 genes; e.g. *ABCA7*, *CCL4L1*). The majority of the 258 genes with decreased expression enriched to GO terms involved in metabolism such as "metabolic process" (FDR-P=3.113e-5; 194 genes; e.g. *CERKL*, *RAD9A*; Fig. 4.6E) and "organic substance metabolic process" (FDR-P=3.113e-5; 186 genes; e.g. *DGAT1*, *GPX4*).

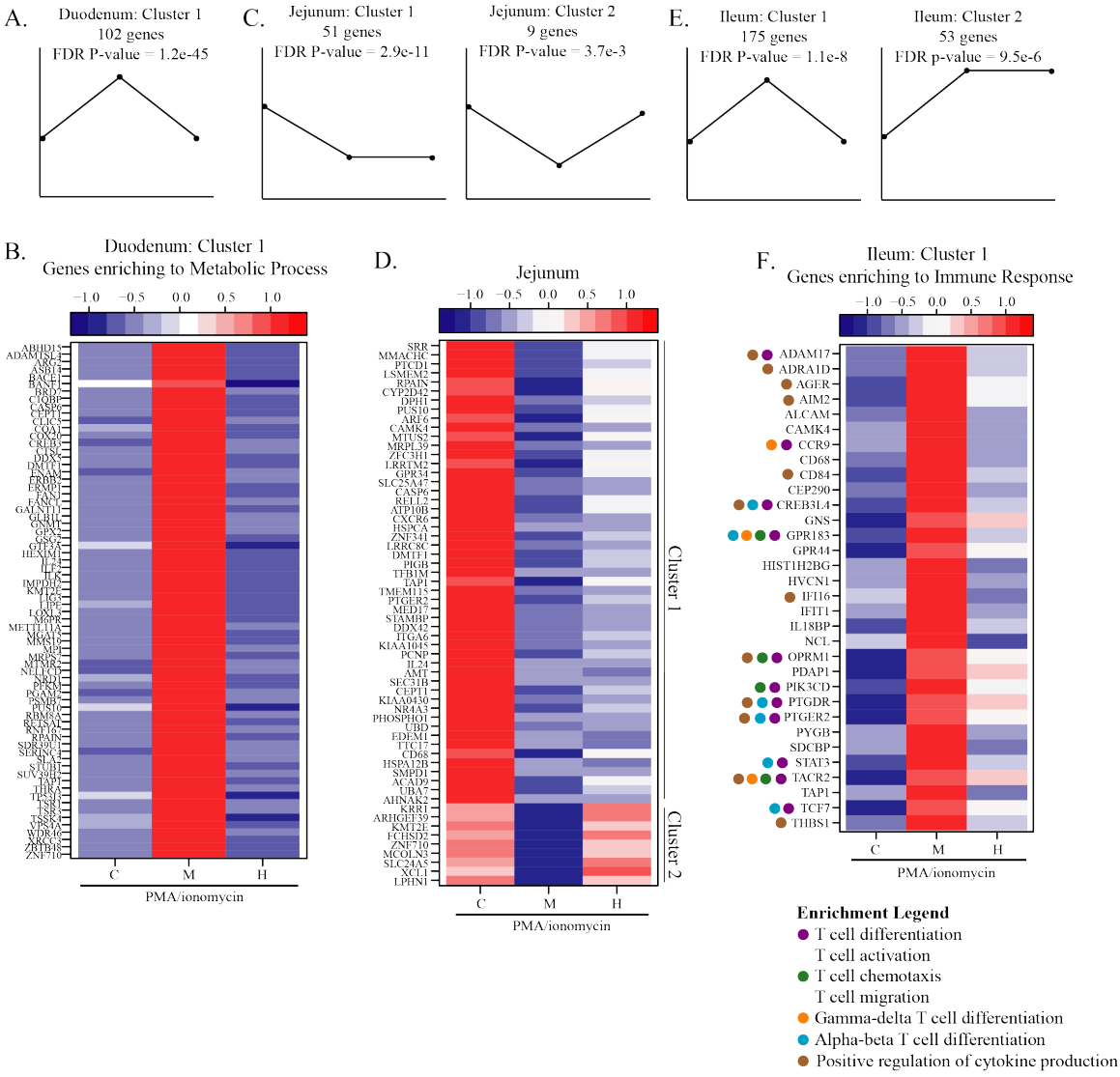

A.
Controls
Response to PMA/ionomycin
Drinkers
Response to PMA/ionomycin
73
55
290
B.
GO Terms
Drinkers Reponse
Number of Upregulated Genes
0
20
40
60
Reg. of cell development
Chemotaxis
Cell differentiation
Cellular component organization
Reg. of signal transduction
0
2
4
6
-log(FDR)
C.
Number of Downregulated Genes
0
50
100
150
200
250
Metabolic process
Organic substance metabolic process
Nitrogen compound metabolic process
T cell migration
Macromolecule metabolic process
3.0
3.5
4.0
4.5
5.0
-log(FDR)
D.
Colon: Upregulated DEG from drinkers
enriching to "Cell differentiation"
−1.0 −0.5 0.0 0.5 1.0 1.5
AMPD1
BZW2
ALCAM
CFTR
COL24A1
CYP26B1
CNTN4
RUNX1T1
FGD2
FAT3
FZD3
GAB3
GFRA3
HSPA5
IFNG
IGSF10
IL2
MAPK10
KRAS
LMX1A
LRFN4
LRRN1
LPHN3
KIRREL
PCSK6
ZMYND8
RIMS2
ROBO2
SHROOM3
SLITRK6
SYNJ1
SEMA3D
ULK2
WDR36
RNF112
Drinkers
PMA/ionomycin
E.
Colon: Downregulated DEG from drinkers
enriching to "Metabolic process"
−1.0 −0.5 0.0 0.5 1.0 1.5
LIMD1
PGAM2
RPS6KB2
EIF3I
SLIRP
LHX4
TEFM
VSIG4
ERCC4
GPX4
CASP6
IL24
GGACT
ALX3
RAB24
GSG2
NME6
TRIM17
ACAD9
WDR46
PIK3CD
PPP1R9B
UBA7
TK1
SH3BGRL3
INO80E
AKIRIN2
PMEL
SRSF12
FBXO31
Drinkers
PMA/ionomycin

(Previous page) Figure 4.6: Gene expression changes in colonic LPLs. (A) Venn diagram depicting the overlap of the total number of differentially expressed characterized human homologs between controls and drinkers in response to PMA/ionomycin. (B) Bar graph displaying the number of characterized human homologs with elevated expression levels in drinkers response to PMA/ionomycin that enriched to the listed Gene Ontology (GO) terms. Line represents the -log(FDR) associated with each GO term. (C) Bar graph displaying the number of characterized human homologs with reduced expression levels in drinkers response to PMA/ionomycin that enriched to the listed Gene Ontology (GO) terms. Line represents the -log(FDR) associated with each GO term. (D) Heatmap of the differentially expressed characterized human homologs from drinkers that enriched to "cell differentiation" (n=35) showing expression levels in individual ethanol-consuming animals before and after PMA/ionomycin. (E) Heatmap of the top 30 characterized human homologs with reduced expression levels in drinkers response to PMA/ionomycin that enriched to "metabolic process" (n=195) showing expression levels in individual ethanol-consuming animals before and after PMA/ionomycin.

Moderate ethanol consumption regulates gene expression in small intestinal LPLs

We next wanted to identify dose-dependent gene expression changes within LPLs following stimulation. To that end, we compared the transcriptomes of controls versus ethanol-consuming animals after PMA/ionomycin stimulation (in contrast to our previous analysis where we compared changes relative to no stimulation background). This analysis revealed 475 DEGs in the duodenum (71 upregulated and 404 downregulated), 158 DEGs in the jejunum (66 upregulated and 92 downregulated), and 808 DEG in the ileum (146 upregulated and 662 downregulated) that were then subjected to STEM analysis. In duodenal LPLs, STEM analysis identified a single cluster of genes, the expression of which increases with non-heavy ethanol consumption then decreases to control levels with heavy drinking (102 genes; Fig. 4.7A). Genes in this cluster enriched primarily to GO term "metabolic process" (FDR-P=0.00516; 80 genes) and included the apoptosis-regulating synthase *PUS10* and the adapter protein *SLA2*, which negatively regulates T-cell receptor signaling (Fig. 4.7B). In jejunal LPLs, STEM analysis identified 2 clusters of genes the expression of which decreases with non-heavy ethanol consumption then remains unchanged or increases to control levels with heavy drinking (60 genes; Fig. 4.7C). Genes in these clusters primarily play a role in lymphocyte activation and cytokine production (e.g. *XCL1*, *FAT10*, *CAMK4*) and regulation of reactive oxygen species metabolism (e.g. *HSPA12B*, *IFNG*; Fig. 4.7D). In ileal LPLs, STEM analysis identified 2 clusters of genes the expression of which increases with non-heavy ethanol consumption then decreases to either control levels (175 genes) or remains unchanged with heavy drinking (53 genes; Fig. 4.7E). There were 32 genes in cluster 1 that functionally enriched to the GO term "immune

response" (FDR-P=0.002071; Fig. 4.7F) that also enriched to pathways involved in "T cell differentiation/activation" (FDR-P=6.548e-5/0.002142; 11 genes; e.g. *ADAP17*, *CCR9*); "T cell chemotaxis/migration" (FDR-P=1.346e-4/0.001261; 4 genes; e.g. *GPR183*, *OPRM1*); and "positive regulation of cytokine production" (FDR-P=0.001261; 12 genes; e.g. *AIM2*, *CD84*). Genes in the second cluster play a role in metabolism and functionally enriched to GO terms such as "lipoxin biosynthetic process" (FDR-P=4.177e-4; e.g. *ALOX12*) and "glutamine catabolic process" (FDR-P=0.00147; e.g. *GLS*).

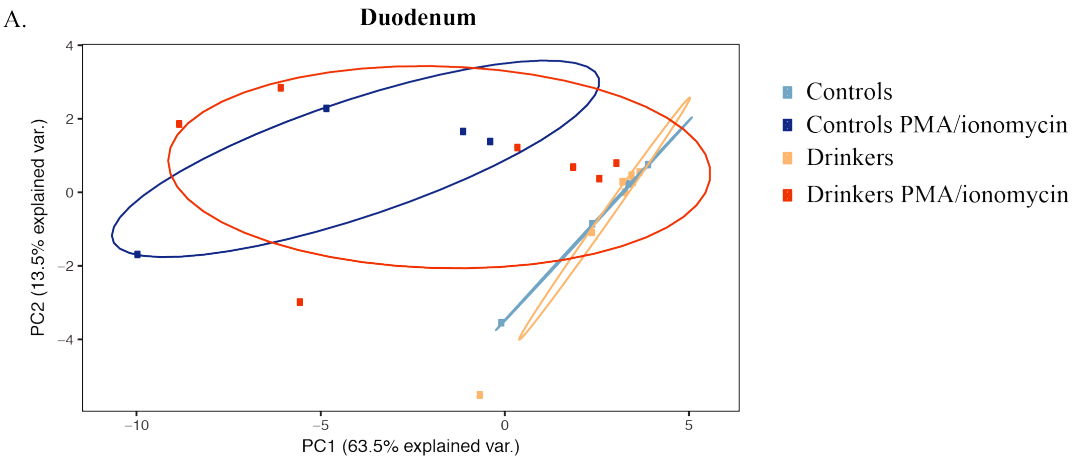

A.
Duodenum: Cluster 1
102 genes
FDR P-value = 1.2e-45
C.
Jejunum: Cluster 1
51 genes
FDR P-value = 2.9e-11
Jejunum: Cluster 2
9 genes
FDR P-value = 3.7e-3
E.
Ileum: Cluster 1
175 genes
FDR P-value = 1.1e-8
Ileum: Cluster 2
53 genes
FDR p-value = 9.5e-6
B.
Duodenum: Cluster 1
Genes enriching to Metabolic Process
−1.0 −0.5 0.0 0.5 1.0
C M H
PMA/ionomycin
D.
Jejunum
Cluster 1
Cluster 2
F.
Ileum: Cluster 1
Genes enriching to Immune Response
ADAM17
ADRA1D
AGER
AIM2
ALCAM
CAMK4
CCR9
CD68
CD84
CEP290
CREB3L4
GNS
GPR183
GPR44
HIST1H2BG
HVCN1
IFI16
IFIT1
IL18BP
NCL
OPRM1
PDAP1
PIK3CD
PTGDR
PTGER2
PYGB
SDCBP
STAT3
TACR2
TAP1
TCF7
THBS1
Enrichment Legend
T cell differentiation
T cell activation
T cell chemotaxis
T cell migration
Gamma-delta T cell differentiation
Alpha-beta T cell differentiation
Positive regulation of cytokine production

(Previous page) Figure 4.7: Moderate consumption uniquely modulates gene expression of small intestinal LPLs. (A) Line graph depicting the dose-dependent dynamics changes in gene expression in stimulated duodenal LPLs. (B) Heatmap of the characterized human homologs from cluster 1 in the duodenum that enriched to "metabolic process" (n=70) showing median expression levels of controls, moderate drinkers, and heavy drinkers after exposure to PMA/ionomycin. (C) Line graphs depicting the dose-dependent dynamics changes in gene expression in stimulated jejunal LPLs. (D) Heatmap of the characterized human homologs from cluster 1 and 2 in the jejunum showing median expression levels of controls, moderate drinkers, and heavy drinkers after exposure to PMA/ionomycin. (E) Line graphs depicting the dose-dependent dynamics changes in gene expression in stimulated ileal LPLs. (F) Heatmap of the characterized human homologs from cluster 1 in the ileum that enriched to "immune response" (n=32) showing median expression levels of controls, moderate drinkers, and heavy drinkers after exposure to PMA/ionomycin. Colored dots represent characterized human homologs that enrich to the listed Gene Ontology (GO) terms in the Enrichment Legend.

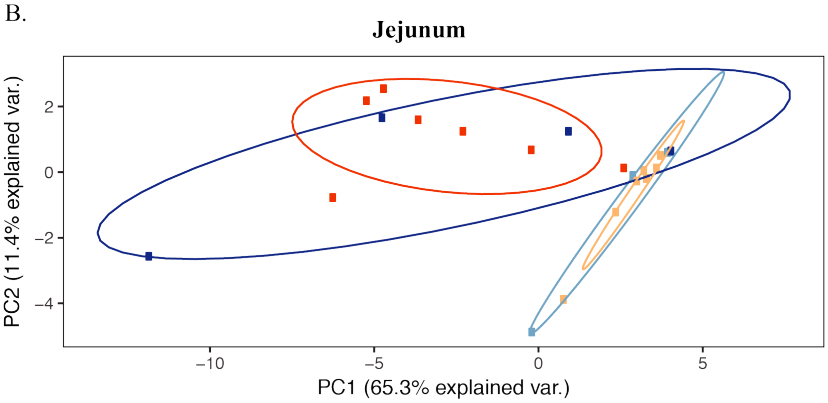
Small intestinal LPL cytokine, chemokine, and growth factor production in response to PMA/ionomycin following chronic ethanol consumption

We next compared soluble factor production by stimulated LPLs isolated from drinkers versus those of controls. Principal component analysis (PCA) showed that profiles of resting and stimulated duodenal LPL obtained from controls formed two distinct clusters while those of ethanol-consuming animals were overlapping, which suggests a greater response in the control group (Fig. 4.8A). Indeed, duodenal LPL isolated from controls showed a significant increase in production of: FGF-basic, IL-1b, IL-10, IL-12, RANTES, IL-17, GM-CSF, EGF, HGF, VEGF, IFN-g, I-TAC, TNF-a, IL-2, IP-10, and MIG in response to PMA/ionomycin stimulation. The duodenal LPL isolated from ethanol-consuming animals showed increased secretion of some of these mediators: FGF-basic, IL-1b, RANTES, IL-17, HGF, I-TAC, TNF-a, and MIG in response to PMA/ionomycin stimulation (Fig. 4.9). Additionally, duodenal LPL isolated from ethanol-consuming animals secreted higher levels of mediators not detected in control LPL cultures: MIP-1a, MIP-1b, IL-5, and IL-8.

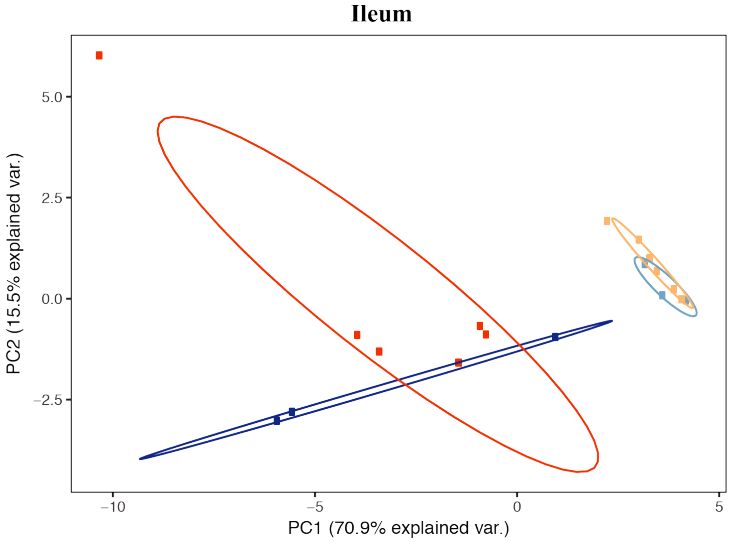
A.


Duodenum
PC2 (13.5% explained var.)
PC1 (63.5% explained var.)
Controls
Controls PMA/ionomycin
Drinkers
Drinkers PMA/ionomycin

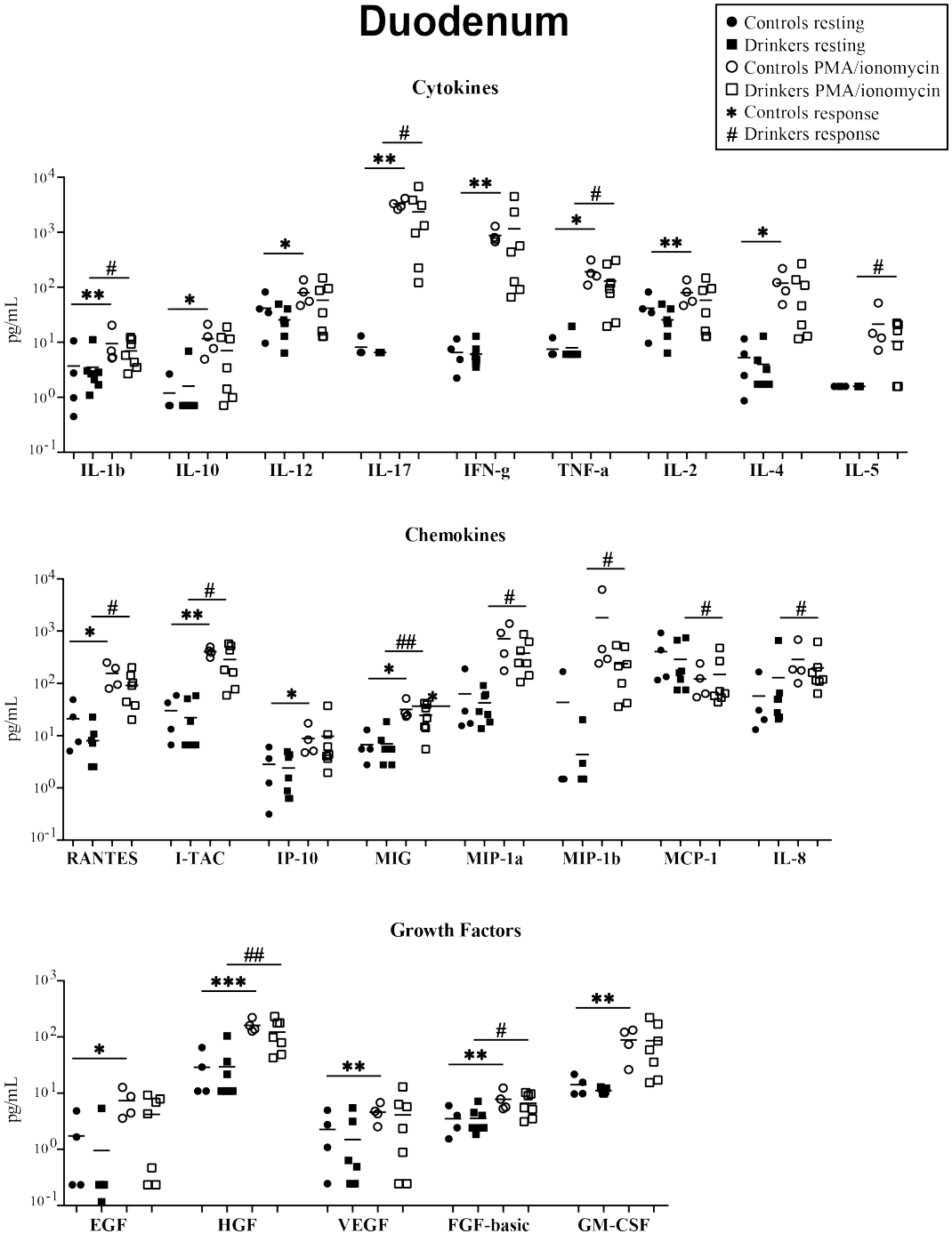
B.


Jejunum
PC2 (11.4% explained var.)
PC1 (65.3% explained var.)

C.

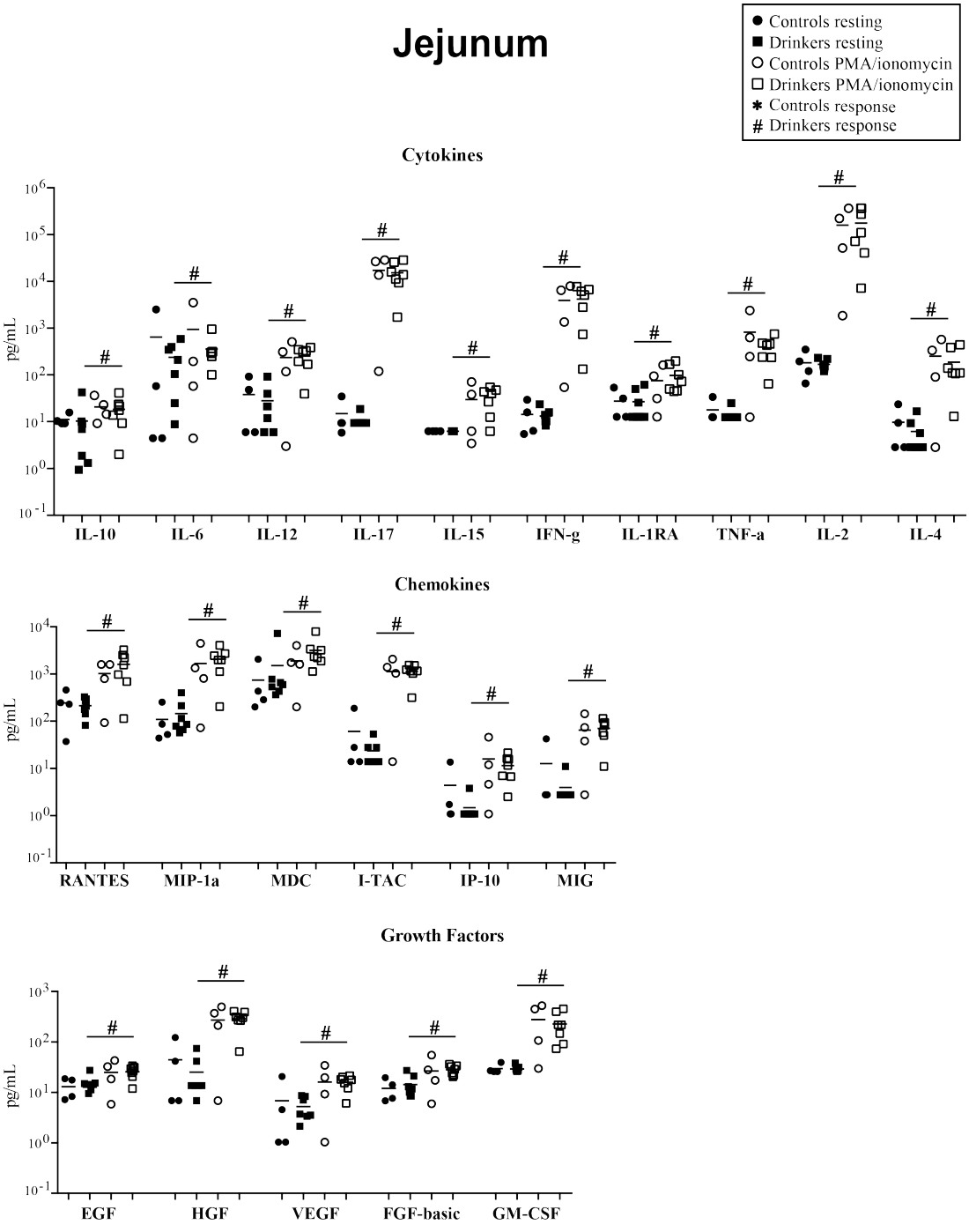

Ileum
PC2 (15.5% explained var.)
PC1 (70.9% explained var.)

(Previous page) Figure 4.8: Alterations in protein secretion by LPLs with ethanol consumption. Principal component analysis (PCA) of protein values to examine differences in (A) duodenal, (B) jejunal, and (C) ileal LPLs isolated from controls and drinkers in their response to PMA/ionomycin.


Duodenum
Controls resting
Drinkers resting
Controls PMA/ionomycin
Drinkers PMA/ionomycin
Controls response
Drinkers response
Cytokines
pg/mL
IL-1b
IL-10
IL-12
IL-17
IFN-g
TNF-a
IL-2
IL-4
IL-5
Chemokines
RANTES
I-TAC
IP-10
MIG
MIP-1a
MIP-1b
MCP-1
IL-8
Growth Factors
EGF
HGF
VEGF
FGF-basic
GM-CSF

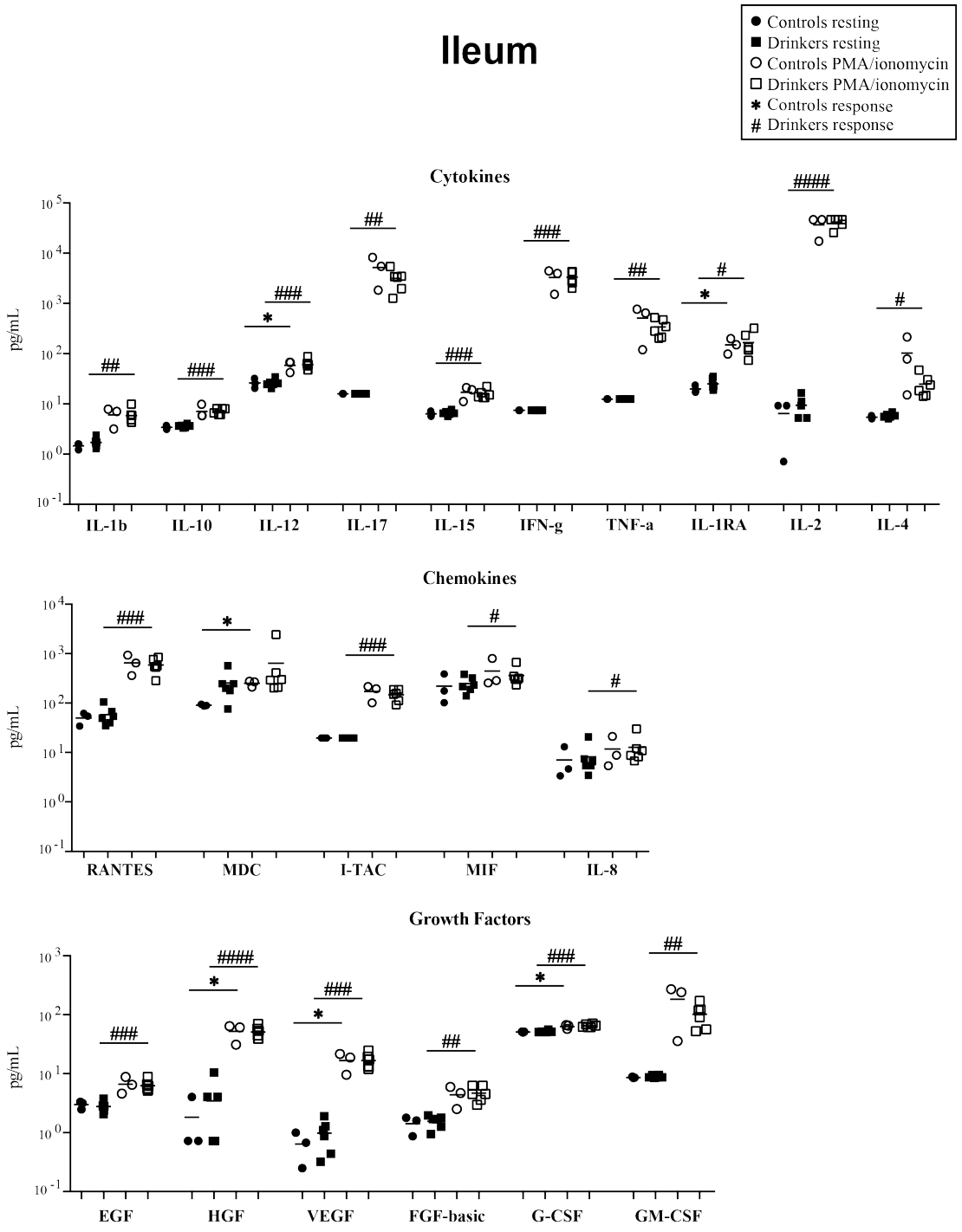
(Previous page) Figure 4.9: Cytokine, chemokine, and growth factor production by duodenal LPL in response to PMA/ionomycin. Scatter plots showing (A) cytokine, (B) chemokine, and (C) growth factor production by duodenal LPL isolated from controls and drinkers before and after exposure to PMA/ionomycin.

In contrast to the duodenum, PCA showed profiles of resting and PMA/ionomycin-stimulated jejunal LPLs from controls overlapped whereas those from ethanol-consuming animals were distinct (Fig. 4.8B). In line with these observations, we detected no significant differences in cytokine, chemokine, or growth factor production in response to PMA/ionomycin stimulation by jejunal LPL isolated from controls. In contrast, the jejunal LPL isolated from ethanol-consuming animals showed significantly increased levels of FGF-basic, IL-10, IL-6, IL-12, RANTES, IL-17, MIP-1a, GM-CSF, IL-15, EGF, HGF, VEGF, IFN-g, MDC, I-TAC, IL-1RA, TNF-a, IL-2, IP-10, MIG, and IL-4 in response to PMA/ionomycin stimulation (Fig. 4.10).


Jejunum
Controls resting
Drinkers resting
Controls PMA/ionomycin
Drinkers PMA/ionomycin
Controls response
Drinkers response
Cytokines
pg/mL
IL-10
IL-6
IL-12
IL-17
IL-15
IFN-g
IL-1RA
TNF-a
IL-2
IL-4
Chemokines
RANTES
MIP-1a
MDC
I-TAC
IP-10
MIG
Growth Factors
EGF
HGF
VEGF
FGF-basic
GM-CSF

(Previous page) Figure 4.10: Cytokine, chemokine, and growth factor production by jejunal LPL in response to PMA/ionomycin. Scatter plots showing (A) cytokine, (B) chemokine, and (C) growth factor production by jejunal LPL isolated from controls and drinkers before and after exposure to PMA/ionomycin.

PCA of immune mediator production by ileal LPL showed that stimulated samples for both controls and ethanol-consuming animals formed distinct clusters (Fig. 4.8C). At the individual analyte level, ileal LPL isolated from controls secreted: G-CSF, IL-12, HGF, VEGF, MDC, and IL-1RA in response to PMA/ionomycin stimulation. On the other hand, ileal LPLs isolated from ethanol-consuming animals secreted: FGF-basic, IL-1b, G-CSF, IL-10, IL-12, RANTES, IL-17, GM-CSF, IL-15, EGF, HGF, VEGF, IFN-g, I-TAC, MIF, IL-1RA, TNF-a, IL-2, IL-4, and IL-8 in response to PMA/ionomycin stimulation (Fig. 4.11). After correction for background, levels of IL-8 were significantly negatively associated with g/kg/day ($r=-0.94$; $p=0.02$).


Ileum
Controls resting
Drinkers resting
Controls PMA/ionomycin
Drinkers PMA/ionomycin
Controls response
Drinkers response
Cytokines
pg/mL
IL-1b
IL-10
IL-12
IL-17
IL-15
IFN-g
TNF-a
IL-1RA
IL-2
IL-4
Chemokines
RANTES
MDC
I-TAC
MIF
IL-8
Growth Factors
EGF
HGF
VEGF
FGF-basic
G-CSF
GM-CSF

(Previous page) Figure 4.11: Cytokine, chemokine, and growth factor production by ileal LPL in response to PMA/ionomycin. Scatter plots showing (A) cytokine, (B) chemokine, and (C) growth factor production by ileal LPL isolated from controls and drinkers before and after exposure to PMA/ionomycin.

Discussion

The intestinal mucosal immune system has one of the most critical tasks, acting as the first line of defense against the various pathogens and insults encountered in the gastrointestinal tract. Chronic alcohol consumption impairs the intestinal mucosal barrier and alters frequency and function of gut-resident immune cells. Intestinal lamina propia lymphocytes (LPLs), which are predominately CD4+ T cells, are major effector cells distributed along the gastrointestinal tract [348], and their functional impairment can significantly impact intestinal homeostasis. Therefore, in this study, we sought to uncover the dose- and site-specific impact of long-term ethanol consumption on the response of LPLs to stimulation. To that end, we used a rhesus macaque model of ethanol self-administration and examined the response of LPLs harvested from all 4 major gut sections after 12 months of ethanol consumption. Using this model, we recently reported changes in the expression of genes involved in protein trafficking, signaling, metabolism, inflammation, and colorectal cancer development in biopsies collected from small and large intestine [349]. Here, we extended our previous study to identify the site- and dose-dependent impact of chronic ethanol consumption on intestinal LPLs and their response to PMA/ionomycin stimulation.

Our two-group analysis did not uncover gene expression differences in resting intestinal LPLs, except for the ileum, which showed a downregulation in genes responsible for the regulation of localization including components of adherens junctions (*ABLIM3*) and regulators of cell motility (*ADAM9*). AUD patients often exhibit defects in nutrient absorption and barrier function, therefore a reduction in the expression of genes responsible

for regulating cellular localization, movement, and transport could further negatively impact the intestinal mucosal barrier. Additional analyses that take into account the variable drinking patterns revealed ethanol dose-dependent changes in LPL gene expression. Specifically, in resting small intestinal LPLs, chronic moderate alcohol consumption impacted the expression of genes critical for chromatin organization; metabolism; and components of the mitochondrial respiratory chain for ATP synthesis and energy harvest.

Following 12 hours of exposure to PMA/ionomycin stimulation, duodenal and jejunal LPLs regardless of drinking status showed a reduction in the expression of genes associated with cell differentiation. Genes upregulated only in duodenal LPLs from the control group play a role in T cell homing to the gut and activation. Genes downregulated in ethanol-consuming animals are important for metabolism. Furthermore, genes involved in calcium ion transport were downregulated only in jejunal LPLs isolated from drinkers indicative of an aberrant response to PMA/ionomycin exposure.

Similar to our findings in gut mucosal biopsies, LPLs isolated from the ileum showed the largest changes in gene expression in response to stimulus. DEGs detected in ileal LPLs from control animals were involved in protein kinase signaling pathways. As described for proximal small intestinal LPLs, expression of genes involved in metabolic processes was significantly reduced in ileal LPLs of ethanol-consuming animals. These genes are important for the response to stress including *HSP90*, *HSF1*, and *NLRC4*. Interestingly, chronic alcohol consumption has been shown to activate the transcription factor HSF1, which leads to the expression of HSP90. Furthermore, *HSP90* mRNA and

protein levels are increased in livers of AUD patients with ALD [350]. Our data suggest the impact of alcohol on heat shock factor expression is tissue-specific and depends on status of liver injury. Expression of genes critical for cell growth was also downregulated in ileal LPLs from ethanol-consuming animals, which suggests impairment in cell division, development, and proliferation.

There were also several common DEGs detected in both controls and drinkers in the ileum. Of the downregulated genes, which also exhibited similar stimulated transcriptional profiles, ileal LPLs isolated from drinkers showed a higher level of expression in several immune genes at rest compared to control LPLs. Surprisingly, these genes were not statistically significant when resting LPLs from drinkers and controls were compared. In contrast, upregulated DEGs common among drinkers and controls showed similar resting transcriptional profiles, while ileal LPLs from drinkers showed higher levels of expression of immune (*PPP3R1*, *ORAI1*), metabolic (*SRR*), proliferative (*NUDT6*), and apoptotic (*PUS10*) genes following PMA/ionomycin, with the exception of *IFNG* which was higher in controls than compared to drinkers.

In the colon, LPLs from both control and drinker groups upregulated genes involved in T cell function including *IL2* and *IFNG* following PMA/ionomycin exposure. Similar to the other gut sections, alcohol consumption resulted in a large reduction in the expression of metabolic genes. The kinase *CERKL*, which regulates apoptosis and has shown to be protective against oxidative stress [351], was downregulated. Cell cycle checkpoint proteins critical for DNA damage repair such as *RAD9A* were also downregulated in colonic LPLs from drinkers. These deficits support the development and

progression of colorectal cancer. Overall, our RNA-Seq results reveal altered gene expression in LPLs associated with aberrant inflammation, cell cycle progression, and cellular metabolism following chronic alcohol consumption.

Small intestinal LPLs showed unique changes in gene expression with moderate alcohol intake in response to PMA/ionomycin. Several genes included those critical for lymphocyte activation and cytokine production including *ADAM17*, *AIM2*, *CD84*, *CAMK4*, *FAT10*, and *XCL1*. Additional studies are warranted to fully understand how chronic moderate ethanol consumption modulates intestinal LPL function.

Our Luminex results suggest LPLs from both drinkers and controls isolated from each of the major segments of the small intestine displayed distinct responses following PMA/ionomycin stimulation. Specifically, duodenal LPLs from controls and drinkers significantly upregulated pro-inflammatory factors including FGF-basic, IL-1b, RANTES, IL-17, HGF, I-TAC, TNF-a, and MIG. However, drinkers failed to upregulate the anti-inflammatory mediator IL-10, as well as IL-12, GM-CSF, EGF, VEGF, IFN-g, IP-10, as well as factors important for proliferation such as IL-2. Furthermore, duodenal LPLs from drinkers uniquely secreted chemokines primarily produced by macrophages (MIP-1a, MIP-1b, IL-8) while LPLs from controls did not. Resting and stimulated LPL profiles of ethanol-consuming animals overlapped, which further suggests a greater response in LPLs isolated from the control group.

In contrast to what we observed in the duodenum, jejunal LPLs from controls generated a small response to PMA/ionomycin exposure, while those isolated from drinkers significantly upregulated the production of FGF-basic, IL-10, IL-6, IL-12,

RANTES, IL-17, MIP-1a, GM-CSF, IL-15, EGF, HGF, VEGF, IFN-g, MDC, I-TAC, IL-1RA, TNF-a, IL-2, IP-10, MIG, and IL-4. In line with these results, PCA showed profiles of resting and PMA/ionomycin-stimulated jejunal LPLs from controls overlapped compared to ethanol-consuming animals, which displayed distinct clusters. The augmented protein secretion suggests a hyperinflammatory response to PMA/ionomycin stimulation following chronic alcohol consumption. However, it is worth noting that the statistical results amongst these groups is likely due to the small sample size of controls available for analysis in the jejunum (4 controls versus 7 drinkers).

Finally, PCA profiles of immune mediator production by ileal LPLs showed that resting and stimulated profiles of controls and drinkers overlapped. Moreover, while there was little variation in resting profiles, stimulated profiles of drinkers showed greater variance compared to controls. Ileal LPLs from controls and drinkers both upregulated G-CSF, IL-12, HGF, VEGF, and IL-1RA in response to PMA/ionomycin stimulation, while LPLs from drinkers additionally secreted FGF-basic, IL-1b, IL-10, IL-12, RANTES, IL-17, GM-CSF, IL-15, EGF, IFN-g, I-TAC, MIF, TNF-a, IL-2, IL-4, and IL-8. The reduction in anti-inflammatory cytokines observed in duodenal LPLs and increase in pro-inflammatory factors produced by small intestinal LPLs following exposure to stimulus likely contributes to the aberrant inflammatory processes observed in the gastrointestinal tract of patients with AUD.

While our RNA-seq analysis suggests a large downregulation of gene expression, our Luminex results suggest significant production of cytokines, chemokines, and growth factors following PMA/ionomycin stimulation. During the 12-hour stimulation assay,

immune mediators were secreted into the LPL culture supernatants. It is likely that following 12 hours of PMA/ionomycin exposure, gene expression is largely downregulated due to dampening of the immune response. The RNA-Seq time point likely represents the termination of the LPL response to PMA/ionomycin. It is also possible that there is a delay in the kinetics of the immune response following PMA/ionomycin exposure with chronic alcohol consumption, but more studies are needed to confirm the kinetics.

In contrast to earlier studies that reported changes in the frequency of LPLs with AUD [141, 142, 144], we did not observe changes in LPL frequency in our macaque model. This discrepancy is likely due to differences in the severity of AUD. Additionally, in contrast to our previous studies, we did not detect a dose-dependent decrease in the production of IL-2, IL-17, TNFα, and IFNγ by colonic T cells [56]. This difference could be due to the fact that our earlier studies measured cytokine production by intracellular cytokine staining following 6 hours of PMA/ionomycin stimulation, whereas in this study we used Luminex following 12 hours of stimulation.

In contrast to our recent gene expression studies using whole tissue biopsies ileum biopsies, we did not detect a large increase in the expression of inflammatory genes in resting ileal LPLs collected from ethanol consuming animals. This differential outcome could be explained by the fact that the LPLs were 60-90% T cells, while our previous *in silico* analysis indicated that the increased expression of inflammatory genes in gut biopsies were derived from B cells as well as some neutrophils and stromal cells [349].

In summary, the studies presented here show that ethanol consumption alters gene expression in intestinal LPLs, notably within the ileum where a large number of metabolic

genes critical for an immune response were dysregulated. Chronic ethanol consumption also led to altered profiles of immune mediator production within small intestinal LPLs that revealed a preference toward a pro-inflammatory phenotype. Limitations of our studies include a limited phenotypic panel, which impeded our ability to determine the minor immune cell subsets contributing to gene expression changes. Finally, we evaluated a limited number of samples and only focused on males, however our findings are significant since we focused on different regions of the gastrointestinal tract in a unique outbred nonhuman primate model. Future studies will focus on gender differences as well as a time course in order to define the kinetics of changes in gene expression and immune mediator production, and their association to the onset of intestinal damage and inflammation seen with AUD.

Chapter 5: Discussion and Future Directions

Approximately 18 million individuals in the United States suffer from AUD, which results in 88,000 deaths and $223.5 billion in health care costs annually. Furthermore, AUD results in perturbations of the intestinal epithelial barrier, mucosal immune responses, and microbiome. Regional and dose-dependent changes in epithelial cell function, immunity, and microbiota are poorly defined since most clinical studies focused on the colon and fecal material obtained from subjects with AUD and ALD. Similarly, rodent models almost exclusively use heavy and binge ethanol intake protocols. The studies described in this dissertation leverage a macaque model of voluntary ethanol self-administration in order to determine the dose-dependent impact of ethanol on vaccination, gut mucosal gene expression and microbial composition, and LPL function.

5.1.1 Modulation of peripheral immunity by alcohol

In chapter 2, we characterized the dose-dependent impact of ethanol consumption on the host response to MVA vaccination using RNA-Seq. We report that chronic heavy ethanol consumption was associated with decreased expression of genes important for host defense against infection and wound healing as well as increased expression of genes associated with development of obstructive lung disease and cancer. In contrast, chronic moderate alcohol consumption was associated with reduced expression of genes involved in neoplasia and increased expression of genes involved in host defense. Furthermore, we examined changes in miRNA expression and found that chronic heavy ethanol consumption altered the expression of several miRNAs whose targets were differentially

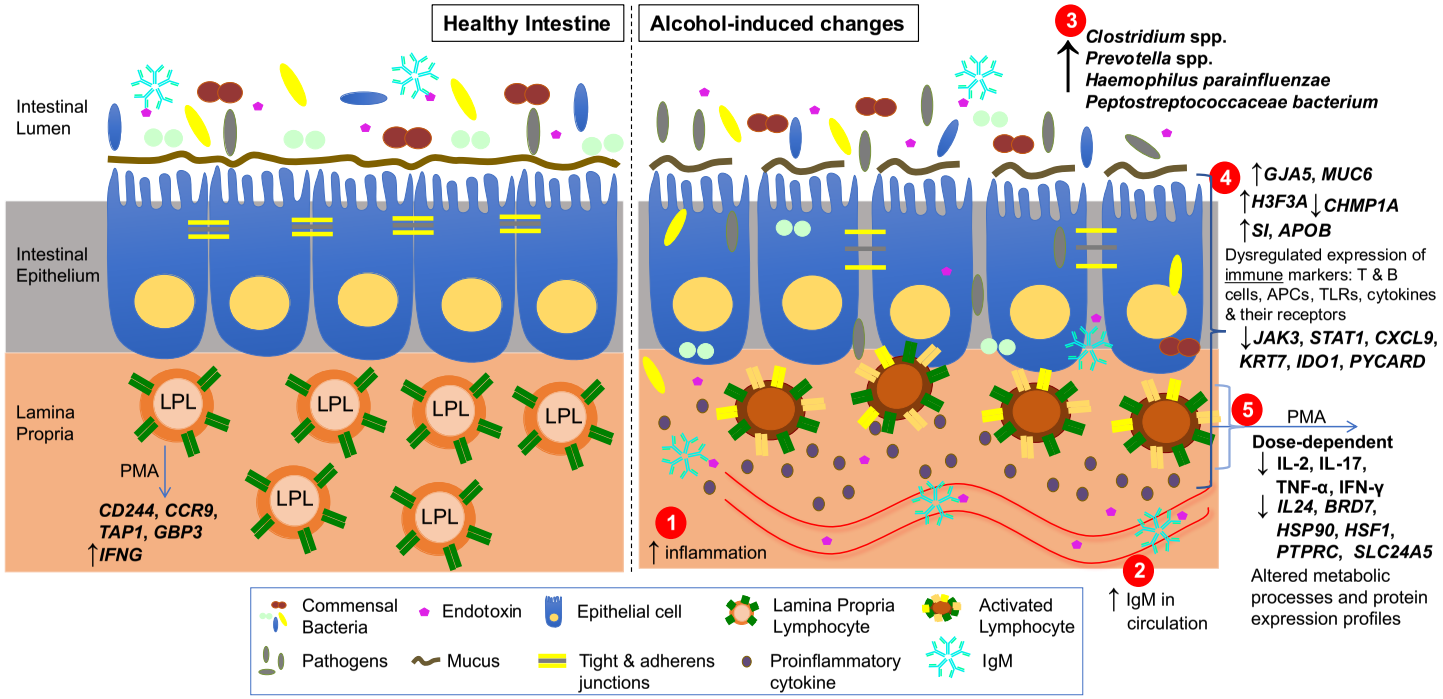
expressed and are involved in cancer progression and immune function. These data provide insight into the mechanisms by which excessive alcohol consumption interferes with protective immune responses, and exacerbates co-morbidities such as poor wound healing, lung disease, and cancer, while moderate consumption improves immunity. Future studies will investigate the mechanisms regulating both mRNA and miRNA gene expression including epigenetic modifications within specific immune cells.

5.1.2 Chronic alcohol consumption alters intestinal immunity

In chapter 3, we characterized the transcriptional profiles and microbiota composition in duodenum, jejunum, ileum, and colon mucosal biopsies from ethanol-consuming and control rhesus macaques (Fig. 5.1). We detected several gene expression changes associated with protein localization, metabolism, inflammation, and colorectal cancer in ethanol-consuming animals. We also found that chronic ethanol consumption led to a dose-dependent decrease in putatively beneficial bacteria, an increase in bacteria associated with inflammation and colorectal cancer, and changes in bacterial metabolic pathways. This was the first study to characterize mucosal gene expression changes and changes in microbiota composition simultaneously in the same biopsies. Given that the ethanol-consuming macaques did not exhibit signs of alcoholic liver disease or colorectal cancer following 12 months of ethanol consumption, our results strongly suggest that gene expression changes precede clinical disease and could serve as early biomarkers of gastrointestinal disease. Future studies will focus on analyzing bacterial communities using shotgun metagenomics and metabolomics. Additionally, the relationship between ethanol-

induced dysbiosis and transcriptional changes needs to be defined. Furthermore, we need to conduct studies aimed at investigating the functional consequences of alterations in specific bacterial species by assessing their impact on barrier function, their ability to translocate, and invade and adhere to epithelial cells. Finally, these studies need to be extended to female macaques to define gender differences in the impact of ethanol self-administration.

In chapter 4, we characterized the ethanol-mediated changes in LPL gene expression and protein secretion from the same four major sections of the gut. In response to PMA/ionomycin, ethanol-consuming animals displayed larger transcriptional changes than control animals, several of which were downregulated metabolic genes. Furthermore, expression of immune mediator and defense genes were increased in ileal LPLs isolated from animals that consumed moderate amounts of ethanol. Profiles of cytokine, chemokine, and growth factor production were distinct in animals that consumed ethanol likely due to the upregulation of pro-inflammatory mediators. Future studies will focus on a time course in order to define the kinetics of these changes in gene expression and their relation to the onset of intestinal damage.


Healthy Intestine
Alcohol-induced changes
Intestinal Lumen
Intestinal Epithelium
Lamina Propria
LPL
PMA
CD244, CCR9, TAP1, GBP3
↑IFNG
1
↑ inflammation
2
↑ IgM in circulation
3
Clostridium spp.
Prevotella spp.
Haemophilus parainfluenzae
Peptostreptococcaceae bacterium
4
↑GJA5, MUC6
↑H3F3A ↓CHMP1A
↑SI, APOB
Dysregulated expression of immune markers: T & B cells, APCs, TLRs, cytokines & their receptors
↓JAK3, STAT1, CXCL9, KRT7, IDO1, PYCARD
5
PMA
Dose-dependent
↓ IL-2, IL-17, TNF-α, IFN-γ
↓ IL24, BRD7, HSP90, HSF1, PTPRC, SLC24A5
Altered metabolic processes and protein expression profiles
Commensal Bacteria
Endotoxin
Epithelial cell
Lamina Propria Lymphocyte
Activated Lymphocyte
Pathogens
Mucus
Tight & adherens junctions
Proinflammatory cytokine
IgM

(Previous page) Figure 5.1: Ethanol-mediated alterations in gastrointestinal immunity in the rhesus macaque model of voluntary ethanol self-administration. Following a 3-month ethanol induction period, rhesus macaques were given "open-access" to both 4% ethanol and water for 22 hours/day and voluntarily self-administered fluids for 12 months. Ethanol consumption led to: 1) increase in lymphoid tissue and infiltration of immune cells; 2) dose-dependent increase in circulating anti-endotoxin IgM levels; 3) increase in bacteria putatively involved in colorectal cancer progression and other inflammatory disorders; 4) dysregulated expression of gut integrity and immune cell makers; and 5) larger transcriptional responses including genes involved in metabolic processes in lamina propria lymphocytes in response to PMA/ionomycin exposure.

5.2 Final thoughts

The data presented in this dissertation provides novel insights into ethanol-mediated dysregulation of both the peripheral and gastrointestinal mucosal immune systems. Molecular mechanisms of the dose-dependent effects of alcohol on the immune system remain poorly understood due to a lack of systematic studies that examine the effect of multiple doses and different time courses. Therefore, this body of data significantly adds to our understanding of the mechanisms underlying ethanol-mediated disruptions of homeostasis and will guide future studies that offer the promise for understanding the complex impact of alcohol on immunity and health.

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