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Summary of the 2018 Alcohol and Immunology Research Interest Group (AIRIG) meeting

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Abstract

On January 26, 2018, the 23rd annual Alcohol and Immunology Research Interest Group (AIRIG) meeting was held at the University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado. The meeting consisted of plenary sessions with oral presentations and a poster presentation session. There were four plenary sessions that covered a wide range of topics relating to alcohol use: Alcohol and Liver Disease; Alcohol, Inflammation and Immune Response; Alcohol and Organ Injury; Heath Consequences and Alcohol Drinking. The meeting provided a forum for the presentation and discussion of novel research findings regarding alcohol use and immunology.

Keywords

Alcohol; Liver disease; Inflammation; Organ injury; Health consequences

Introduction

Alcohol use is prevalent in the United States. According to a 2015 survey of individuals 18 and older, 56% reported drinking in the past month (Substance Abuse and Mental Health Services Administration, 2015a), and 26.9% reported binge drinking in the past month (Substance Abuse and Mental Health Services Administration, 2015b). Furthermore, misuse of alcohol places a large stress on the United States economy, costing roughly 249 billion dollars (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015), while, in itself, alcohol is the third leading cause of preventable death in the United States (Mokdad, Marks, Stroup, & Gerberding, 2004). Based on alcohol's wide use, economic burden, and associated morbidity, understanding of how alcohol induces detrimental physiologic changes is imperative. From an imunologic perspective, alcohol has displayed potent effects in dampening as well as exaggerating the innate and adaptive immune responses in a context-dependent manner (Lau, Szabo, & Thomson, 2009; Molina, Happel, Zhang, Kolls, & Nelson, 2010). The 2018 AIRIG meeting allowed researchers to share their ideas and continue their goal of deciphering the mechanism of alcohol's effects with a particular focus on immunology. This year the meeting was divided into four plenary sessions that focused on alcohol with respect to liver disease, the immune response, organ injury, and health consequences.

The 2018 AIRIG meeting began with opening remarks from Drs. Elizabeth J. Kovacs (University of Colorado Denver) and Richard Schulick (University of Colorado Denver). They highlighted the importance of studying the intersection of alcohol and immunology, and thanked the attendees for their continued research in this field. Additionally, they acknowledged the National Institute on Alcohol Abuse and Alcoholism (NIAAA) for its financial support of AIRIG meetings through R13 meetings grants and for sending Dr. Li

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Lin to attend the meeting. Dr. Lin described NIAAA funding opportunities available to trainees and junior investigators and encouraged them to apply. Altogether, they emphasized the dedication of alcohol researchers that has led to numerous discoveries that have improved the health of patients and advanced our understanding of alcohol's physiologic effects.

Session 1: Alcohol and Liver Disease

The chairs of the first session, Dr. Geoffrey Thiele (University of Nebraska Medical Center) and Suhas Sureshchandra (University of California, Riverside), invited Dr. Hidekazu Tsukamoto from the University of Southern California to begin the session with his talk titled "Is alcoholic hepatitis (AH) a disease of infection?" Alcoholic hepatitis is a clinically unique and important spectrum of alcoholic liver disease with high mortality and limited therapeutic options. Polymorphonuclear leukocyte (PMN) infiltration is a hallmark of AH, yet neither the mechanism nor pathologic significance of this inflammation is known (Gao & Tsukamoto, 2016; Mardrekar, Bataller, Tsukamoto, & Gao, 2016). In particular, it is not known whether PMNs infiltrating into the liver parenchyma is causal for AH or merely a consequence of the underlying disease process. In fact, the treatments designed and implemented to suppress inflammation, to date, have had limited success. There are two primary reasons why PMNs infiltrate into the tissue parenchyma: necrosis and infection. Using a clinically relevant mouse model of AH (Lazaro et al., 2015), the Tsukamoto laboratory has recently identified the Caspase-11 (CASP11)-Gasdermin-D (GSDM-D) pathway, which is uniquely activated in AH but not in preceding chronic alcoholic steatohepatitis (Khanova et al., 2018). This pathway is induced by excessive intracellular levels of the pathogen-associated molecular pattern lipopolysaccharide (LPS), commonly caused by a Gram-negative bacterial infection, and triggers a form of cell death called pyroptosis. Pyroptosis is a programmed and lytic cell death that releases intracellular pathogen-associated molecular patterns/bacteria and damage-associated molecular patterns to the local microenvironment, inciting PMN inflammation. If excessive, endotoxemia and septicemia may ensue, leading to systemic inflammatory response syndrome, the major complication of AH. Isolated hepatic macrophages and hepatocytes from male C57BL/6 mice with AH show activation of GSDM-D in both cell types, suggesting that both are subjected to pyroptosis in AH. Indeed, activation of Caspase-4 (orthologue of CASP11) and GSDM-D is also robust in liver tissues of AH patients. In male mice with whole body knockout for CASP1/11 (B6N.129S4 (D2)- Casp4^{tm1Yuan}I*J*), GSDM-D activation, hepatocyte pyroptosis, liver bacterial load, and liver injury are all reduced in the AH model. Conversely, the deficiency of 1L-18, a key anti-microbial cytokine, upregulates CASP11-GSDM-D activation and worsens liver bacterial load and injury under the AH regimen. Further, hepatocyte-specific expression of active GSDM-D via an adeno-associated virus vector increases pyroptotic death of hepatocytes and PMN inflammation in the AH mice. Based on these results, Dr. Tsukamoto proposed that bacterial infection may be upstream of the activated CASP11/4-GSDM-D pathway, pyroptosis, and PMN infiltration in AH. This novel notion of the pyroptotic pathway may suggest a need for a paradigm shift for therapeutic approaches for AH.

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The next speaker of the session was Dr. Bin Gao from the NIAAA. His talk was titled "Mitochondrial DNA (mtDNA) -enriched extracellular vesicles promote acute-on-chronic alcoholic neutrophilia and hepatotoxicity". Neutrophil infiltration is a hallmark of alcoholic steatohepatitis and AH, but the underlying mechanisms are not clear. Rodent models with chronic ethanol feeding have been used to study alcoholic liver injury for more than three decades, but few intrahepatic neutrophils are detected in these models. Recently, the Gao laboratory developed a mouse model of chronic-plus-binge ethanol feeding, in which binge alcohol consumption induced neutrophilia, hepatic neutrophil infiltration, and liver injury in chronically ethanol-fed mice (Bertola, Mathews, Ki, Wang, & Gao, 2013). Using this model, they found that binge ethanol significantly activated hepatic endoplasmic reticulum (ER) stress and inflammatory responses, and elevated serum levels of mtDNA-enriched microparticles (MPs) in chronically ethanol-fed female C57BL/6 mice (Cai et al., 2017). Genetic deletion of ER stress-related genes (whole body knockout of c-Jun N-terminal kinase [*Jnk*], and transcription factor C/EBP homologous protein [*Chop*], or hepatocyte specific knockout of protein kinase RNA-like ER kinase [*Perk*]) or inhibition of ER stress with chemical inhibitors, reduced levels of circulating mtDNA-enriched MPs, neutrophilia, and liver injury in mice with chronic-plus-binge ethanol challenges (Cai et al., 2017). Hepatocyte-specific protein cytochrome P450, family 2, subfamily E protein was detected in MPs isolated from ethanol-fed mice, suggesting that hepatocytes were the main source of mtDNA-enriched MPs after ethanol feeding (Cai et al., 2017). Finally, administration of mtDNA-enriched MPs isolated from chronic-plus-binge ethanol-fed mice caused neutrophilia in mice (Cai et al., 2017). Collectively, the data from these experimental mouse models suggested that chronic-plus-binge ethanol challenge activates hepatic ER stress-dependent mtDNA-enriched MP release, leading to neutrophilia and liver injury. In addition, the Gao laboratory examined 300 human subjects with excessive alcohol use (EAU) and stratified them into two groups: EAU with or without recent excessive drinking. Their analysis revealed that subjects with EAU with recent excessive drinking had markedly higher levels of circulating neutrophils, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and circulating mtDNA-enriched MPs than subjects with EAU without recent drinking and healthy controls (Li et al., 2017). The levels of circulating neutrophils correlated positively with serum levels of ALT and AST (Li et al., 2017). Furthermore, the number of MPs and levels of mtDNA in circulation correlated positively with circulating neutrophils in subjects with EAU with recent excessive drinking but not in those subjects with EAU without recent drinking (Cai et al., 2017). These data suggested that binge drinking also elevated circulating mtDNA and neutrophils in alcoholics, which likely contributes to liver injury in individuals exhibiting this pattern of alcohol misuse.

Dr. Lin Jia from the University of Texas Southwestern Medical Center at Dallas gave the next talk, "Hepatocyte Toll-like receptor 4 (TLR4) regulates alcoholic fatty liver disease in mice". 1ncreasing lines of evidence suggest that inflammation is an essential factor contributing to the initiation and progression of alcoholic liver disease (ALD). Specifically, elevated endotoxin/LPS levels have been reported in subjects and rodent models with ALD. Circulating LPS interacts with its cell membrane receptor TLR4, leading to the activation of the signaling pathway and the generation of pro-inflammatory cytokines. Indeed, both antibiotic-treated and global TLR4 knockout mice are protected from ALD. Primary

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hepatocyte and hepatoma cell lines express TLR4. In addition, the Jia laboratory (Jia et al., 2014) and others (Uchimura et al., 2014) have demonstrated a physiologically important role of hepatocyte TLR4 in regulating tissue and systemic inflammation, as well as liver fat accumulation. Collectively, these data support a model in which hepatocyte TLR4 mediates the development of ALD. To answer this question, Dr. Jia used two unique mouse models that selectively delete TLR4 (*Tlr4*^{LKO}) or reactivate TLR4 (*Tlr4*^{LoxTB} *X Alb-Cre*) in hepatocytes, respectively. Male mice on a mixed genetic background (B6/129) were fed a liquid diet containing either 5% ethanol or pair-fed a control diet for 4 weeks. They found that mice lacking hepatocyte TLR4 (*Tlr4*^{LKO}) had significantly decreased plasma ALT and AST concentrations and reduced hepatic triglyceride contents after chronic alcohol intake. In addition, ethanol-fed *Tlr4*^{LKO} mice showed greatly reduced inflammation in the adipose tissue and circulation. In contrast, mice with reactivated *Tlr4* expression only in hepatocytes but not in other tissues (*Tlr4*^{LoxTB} *X Alb-Cre*) had significantly increased hepatic triglyceride content, elevated thiobarbituric acid-reactive substance levels, and enhanced mRNA expression of inflammatory genes in the liver after 4 weeks of alcohol feeding. Taken together, these findings suggest that hepatocyte TLR4 plays a key role in regulating alcohol-induced early-stage liver injury and hepatic triglyceride accumulation in mice.

The final speaker of the session was Jacob McGowan, a graduate student in the laboratory of Dr. Geoffrey M. Thiele from the University of Nebraska Medical Center. The title of his talk was "*In vitro* comparison of ethanol metabolism in precision-cut liver slices from C57BL/6, BALB/c, DBA/2J and 129S1/SvlmJ mice". A number of studies have examined the metabolism of ethanol in mice from many different genetic backgrounds (Yoneyama, Crabbe, Ford, Murillo, & Finn, 2008). These studies evaluated the response of ethanol both *in vivo* and *in vitro* on a cellular level (Fidler et al., 2012; Rocco, Compare, Angrisani, Sanduzzi Zamparelli, & Nardone, 2014; Yoneyama et al., 2008). A number of problems exist due to the variance in consumption between strains and maintaining viable and pure cell cultures. In addition, these previous *in vitro* studies only evaluated the cells in isolation and not in combination. Precision-cut liver slices provide a novel method for the systematic analysis of many different strains of mice with respect to how they metabolize ethanol indiscriminately of how much ethanol they would consume *in vivo*. Precision-cut liver slices from female C57BL/6, BALB/c, DBA/2J, and 129S1/SvlmJ mice were evaluated for their responses to ethanol at 25 mM over a 72-h period. Biological outputs of adenosine triphosphate, lactate dehydrogenase (LDH), IL-6, tumor necrosis factor alpha (TNFα), and lipid peroxidation levels by thiobarbituric acid-reactive substance assay were determined. Gas chromatography was used to analyze for ethanol and acetaldehyde. Lipid accumulation in ethanol-treated frozen tissue was evaluated by Oil Red O staining. Cell viability, as measured by adenosine triphosphate levels, remained consistent over the 72-h period for all strains of mice. However, LDH levels increased minimally in the C57BL/6, BALB/c, and DBA strains of mice at 24 h compared to the LDH levels of the 129S1 strain, which increased considerably during the 48 h, suggesting significant cell death. Oil Red O staining of precision-cut liver slices showed an increase in fat accumulation that was present across all time points in all strains of ethanol-fed mice. Triglyceride levels were also increased in each strain exposed to ethanol. Yet, 129S1 and DBA strains showed substantial increases in fat and triglycerides as compared to C57BL/6 or BALB/c strains of mice. Furthermore, the

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129S1 strain was particularly vulnerable to ethanol, as determined by LDH release. In conclusion, the results revealed that livers from different strains of mice exhibit differential metabolism of ethanol, suggesting that some strains may be more suited to study specific pathways of liver alcohol metabolism.

Session 2: Alcohol, Inflammation and Immune Response

The second session, “Alcohol, Inflammation and Immune Response”, chaired by Dr. Samantha Yeligar (Emory University) and Dr. Sarah Jolley (Louisiana State University Health Sciences Center) began with a presentation by Dr. Craig Coopersmith from Emory University. The title of his talk was “Chronic alcohol and sepsis — molecular and cellular insights”. Both alcohol use disorder and sepsis are independently associated with increased mortality. Further, septic patients with alcohol use disorder have a marked increase in mortality compared to septic patients without this chronic co-morbidity. The Coopersmith laboratory studies potential mechanisms for why mortality is disproportionately increased by the combination of alcohol and sepsis. To study this, they first developed a model of chronic alcohol usage followed by sepsis. Mice were randomized to receive either alcohol or water for 12 weeks and then subjected to cecal ligation and puncture. Alcohol/septic mice had a significantly higher mortality than water/septic mice. An extensive survey demonstrated that while both alcohol in isolation and sepsis in isolation impacted nearly every organ in the body, the combination of alcohol/sepsis disproportionately impacted gut integrity and the immune system (Yoseph et al., 2013). Specifically, alcohol/septic mice had elevated intestinal epithelial apoptosis, decreased crypt proliferation, and shortened villus length. Further, alcohol/septic mice had higher intestinal permeability as well as decreased intestinal tight junction (TJ) proteins, zonula occludens-1, and occludin. Additionally, there was a marked upregulation of TNFα and interferon gamma production following *ex vivo* stimulation in splenic $CD4^+$ T cells in alcohol/septic mice. Natural killer cell frequency was also decreased in both the spleen and bone marrow of alcohol/septic mice. The importance of the changes in gut integrity was assayed by giving both alcohol/septic and water/septic mice systemic epidermal growth factor (EGF) (Klingensmith et al., 2017). When given to alcohol/septic mice, EGF improved gut integrity to levels seen in water/septic mice without EGF. Specifically, EGF improved gut permeability, apoptosis, proliferation, and villus length, although the TJs altered by EGF were different from the TJs impacted by sepsis in isolation. Importantly, this was associated with an improvement in mortality, as survival from alcohol/septic+EGF mice was improved from alcohol/septic mice and similar to water/septic mice. The immune effects of alcohol/sepsis were then assayed beyond the original 24 h from their initial study (Margoles et al., 2016). Alcohol/septic mice delayed the kinetics of CD69 (activation marker) on naïve $CD4^+$ T cells while prolonging CD69 expression on memory T cells when assayed at both 24 and 72 h. Alcohol/sepsis also delayed the increase in the frequency of O-glycosylated $CD43^+$ memory $CD4^+$ T cells. The expression of the O-glycosylated form of CD43 is indicative of effector status and is associated with cytolytic function (Margoles et al., 2016). Finally, alcohol/septic mice had a delayed increase in $CD69^+CD43^+CD4^+$ T cells in both naïve and memory cells. Altogether, the results indicate that the combination of alcohol and sepsis lead to deleterious outcomes that are mediated by changes in the gut and immune system.

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The next presenter of the session was Dr. Maria Camargo Moreno, a postdoctoral fellow in the laboratory of Dr. Erin Lowery, from Loyola University of Chicago. The title of her talk was “Antimicrobial peptides, cytokines, and alcohol exposure in lung donors”. The immune system of the lungs decreases in function under excessive alcohol consumption in response to an infection (Moss & Burnham, 2006). In regard to lung transplants, lungs that are donated by excessive alcohol consumers have a higher risk of primary graft dysfunction (Lowery et al., 2014; Pelaez et al., 2015). Dr. Camargo Moreno studies the way the immune response is altered in lung donors who consume alcohol excessively and have an infection as identified by culture positive growth. Her primary focus is on antimicrobial peptides, a primitive component of the innate immune response. She examined three antimicrobial peptides that are known to be expressed in the lungs: cathelicidin (LL-37), α defensins 1–3, and β defensin 2. Based on the Loyola University Hospital lung donor cohort from 2014 to 2017, she compared two groups of lung donors: excessive drinkers (N = 30) vs. nondrinkers (N = 15); recent drinkers (N = 43) vs. no recent drink (N = 26). Excessive drinkers were defined as those that met the Centers for Disease Control and Prevention guidelines for excessive drinkers (greater than 7 drinks per week for females and greater than 14 drinks per week for males), while also having a phosphatidyl ethanol concentration greater or equal to 84 ng/mL. Nondrinkers were defined by a negative alcohol history and negative alcohol biomarkers. Recent drinkers were defined as having a positive blood alcohol content or positive phosphatidyl ethanol measure. Negative blood alcohol content or negative phosphatidyl ethanol measurement was used to determine the “no recent drink” category of lung donor samples. There was an increase in LL-37 in culture-positive samples of excessive drinkers ($p = 0.079$), but not in culture-negative samples. The LL-37 mRNA levels did not differ between excessive drinkers vs. nondrinkers. Cytokine levels of 1L-12, monocyte chemo-attractant protein 1, and interferon gamma ($p = 0.08$) were decreased in excessive drinkers with a positive culture. Comparing these cytokine levels with those of the culture-negative results showed that there was an absence of increased cytokine levels in response to the presence of an infection, compared to nondrinkers. In the recent drinks vs. no recent drinks study samples, there was no statistical difference between age, gender, and smoking. There was a higher percentage of donor lungs with infection and a greater percentage who died from blunt trauma in the recent drink category. The levels of LL-37 were significantly increased in culture-positive samples of recent drinkers compared to the no recent drink category. There were no differences in the protein levels or gene expression of α defensins 1–3, and β defensin 2. Altogether, these preliminary findings reveal that not all lung antimicrobial peptides are affected by alcohol exposure. Furthermore, in the presence of lung infection, lung concentrations of LL-37 and other immune signals are impacted by excessive and recent alcohol exposure in donors.

Holly Hulsebus, a PhD student in the laboratory of Dr. Elizabeth Kovacs, from the University of Colorado Denver, gave the next presentation titled “Alcohol, aging and the pulmonary immune response to *Streptococcus pneumoniae* infection”. The global aging/elderly population (aged >65 years) is increasing and expected to double by 2050. A common behavior in the elderly is alcohol consumption, with 40% consuming 1–2 drinks/day on average 3 days a week (Blazer & Wu, 2009). Even though aged individuals typically drink less than their younger counterparts, the negative health effects of alcohol

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may be more potent due to slower metabolism. The elderly are further affected by "inflamm-aging," which is characterized by low-grade chronic systemic inflammation present even in healthy individuals. Similarly, alcohol misuse correlates with increased systemic inflammation and reduced cell-mediated immunity, which may further contribute to a persistent inflammatory environment (Goral, Karavitis, & Kovacs, 2008). The elderly experience a greater incidence of infection, and increased morbidity and mortality after infection, with otherwise innocuous pathogens such as *Streptococcus pneumoniae* (Stupka, Mortensen, Anzueto, & Restrepo, 2009); however, the effect of alcohol and aging combined has not been investigated. To test the effect of alcohol and advanced age on the pulmonary immune response to *S. pneumoniae,* their laboratory gave young (4–6 months) and aged (19–22 months) female BALB/c mice ethanol by oral gavage for 3 days (1.25 g/kg ethanol; blood alcohol was 50–65 mg/dLat 30 min) followed by intra-nasal infection with 3×10^5 colony-forming units of *S. pneumoniae*. The results indicated that at 24 h after infection, young vehicle-treated mice had focal accumulation of leukocytes in bronchioles that was less apparent in ethanol-exposed young mice and both aged groups. Additionally, aged mice had significantly lower pulmonary expression of the pro-inflammatory genes chemokine (C-X-C motif) ligand 1 and *Il-6* compared to young vehicle-treated mice (33% and 29% reduction, respectively), and this was further lowered when aged mice were given ethanol before infection (66% and 39% reduction, respectively); similar trends were seen with chemokine (C-X-C motif) ligand 2 (*Cxcl2*). Age alone had no effect on lung expression of *Cxcl2* at this time point; however, ethanol exposure prior to infection reduced *Cxcl2* expression in young mice by 17% and in aged mice by 36%, compared to young vehicle. Interestingly, aged mice had a significant increase in expression of the anti-inflammatory cytokine *Il-10* in the lung following infection (2.5-fold higher compared to young vehicle), which was lost in aged animals given ethanol. Lastly, there was no difference in lung bacterial burden at 24 h between young and aged mice given ethanol prior to infection. Future experiments aim to determine the mechanisms leading to impaired innate immune function associated with alcohol and advanced age following infection.

The next talk of the session was titled "Alcohol use promotes intestinal infection in patients with inflammatory bowel disease", presented by Paulius Kuprys, an MD/PhD student in the laboratory of Dr. Mashkoor Choudhry from Loyola University of Chicago. Inflammatory bowel diseases (1BD) affect more than 1.5 million individuals in the United States. There are two forms of 1BD: Crohn's disease (CD) and ulcerative colitis (UC). The disease process of CD is characterized by discontinuous areas of intestinal inflammation throughout the gastrointestinal tract, while UC is characterized by a continuous mucosal ulcer confined to the large intestine. Patients diagnosed with 1BD have periodic episodes of disease reactivation, which produces severe abdominal discomfort and bloody diarrhea, often requiring hospitalization. Certain foods in a patient's diet have been implicated in flares of IBD, which has led physicians to recommend the patients to avoid these foods. One of these foods is alcohol (Brown, Rampertab, & Mullin, 2011); however, only a few studies have addressed the immediate impact of alcohol in the setting of IBD. One study found that UC patients with higher daily alcohol consumption were more likely to have a UC flare (Jowett et al., 2004) and another study found that alcohol drinkers with IBD had worsening of their gastrointestinal symptoms (Swanson, Sedghi, Farhadi, & Keshavarzian, 2010). To further

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assess the effects of alcohol use in the setting of IBD, Paulius conducted a retrospective analysis of a patient database examining outcomes of patients with documented alcohol use who were admitted specifically for 1BD, UC, or CD. Data were obtained from the Healthcare Cost and Utilization Project State Inpatient Databases for New York and Florida from 2009 to 2013. Using the International Classification of Diseases, 9th Revision, Clinical Modification diagnosis codes, Paulius examined patients admitted for IBD, UC, or CD that were then stratified based on whether they had a documented history of alcohol use (+A) or did not (−A). Within each disease group, the +A and −A patients were propensity matched 1:1 for demographics and various comorbidities. *Clostridium difficile* intestinal infection (2% vs. 2.6%), poorly defined intestinal infection (0.4% vs. 0.8%), and overall intestinal infections (2.6% vs. 3.9%) were increased in the IBD +A patients compared to the IBD −A patients. Poorly defined intestinal infections (0.6% vs. 1.5%) were increased in the UC +A patients relative to the UC −A patients, while the CD +A patients had increased intestinal infections of all types (2.1% vs. 3.1%) compared to the CD −A patients. Additionally, compared to the −A patients, the +A patients received significantly more antibiotic injections in all groups (IBD: 3.1% vs. 5.8%; UC: 3.8% vs. 5.5%; CD: 3.4% vs. 5.8%). These findings indicate that alcohol use may produce adverse effects in patients admitted for IBD (Cannon et al., 2018).

Session 3: Alcohol and Organ Injury

The next session titled "Alcohol and Organ Injury" was chaired by Dr. Erin Lowery (Loyola University Chicago) and Dr. Boris Castillo (Florida International University). They invited Dr. Avtar S. Meena, a postdoctoral fellow working with Dr. Radhakrishna Rao from the University of Tennessee Health Science Center Memphis to present his talk "Transient receptor potential vanilloid-6 (TRPV6) -deficient mice are resistant to ethanol-induced disruption of colonic epithelial tight junctions, mucosal barrier dysfunction and liver damage". Dr. Meena began by explaining that chronic alcohol consumption is known to cause ALD (Mann, Smart, & Govoni, 2003); however, only about 20% of alcoholics develop liver disease. The reason for this is not known. He postulated that disruption of intestinal epithelial TJs, gut barrier dysfunction, and endotoxemia play an important role in the pathogenesis of ALD. Their laboratory showed that Ca^{2+} influx and the TRPV6 channel play a crucial role in ethanol- and acetaldehyde-induced TJ disruption and barrier dysfunction in Caco-2 cell monolayers (Gangwar et al., 2017; Samak et al., 2016). Therefore, Dr. Meena hypothesized that deletion of the TRPV6 gene would block ethanol-induced colonic barrier dysfunction and endotoxin absorption *in vivo* and attenuate ethanol-induced liver damage. In his experimental strategy, adult female wild type (WT) and *Trpv6*$^{-/-}$ whole body knockout B6/129 mice were fed 1–6% ethanol (0% 2 days, 1% 2 days, 2% 2 days, 4% 1 week, 5% 1 week, and 6% 1 week) in a Lieber-DeCarli liquid diet; control mice were pair-fed with isocaloric maltodextrin. Dr. Meena showed that plasma-to-luminal flux of fluorescein isothiocyanate-inulin in the distal colon of WT mice was significantly elevated by ethanol feeding, and this effect of ethanol was blocked in the distal colon of *Trpv6*$^{-/-}$ mice. In addition, plasma LPS was elevated by ethanol feeding in WT mice but not in *Trpv6*$^{-/-}$ mice. Ethanol feeding resulted in reduced localization of TJ proteins (occludin and zonula occludens-1) in WT mice, while this effect was blocked in *Trpv6*$^{-/-}$ mice. Furthermore, ethanol feeding reduced the levels of adherens junction proteins (E-cadherin

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and β-catenin) at the epithelial junctions of distal colon in WT mice, and this effect was unaltered in *Trpv6*$^{-/-}$ mice. In terms of inflammation and oxidative stress, ethanol feeding increased cytokine mRNA expression (*Tnfα, Il-6,* chemokine (C-C motif) ligand 5, monocyte chemoattractant protein 1, *Cxcl2*) (Kawaratani et al., 2013), and increased oxidized thiol proteins in WT mice, compared to *Trpv6*$^{-/-}$ mice. These oxidative changes could be attributed to the significantly reduced mRNA levels of antioxidants (Thioredoxin1, superoxide dismutase 1, superoxide dismutase 2) in the colon of ethanol-fed WT mice compared to *Trpv6*$^{-/-}$ mice. Remarkably, F-actin fluorescence was low in ethanol-fed WT mice, which was associated with dramatic elevation of phospho-src. These were blocked in *Trpv6*$^{-/-}$ mice. These findings suggest a mechanism for TRPV6 in TJ disruption of the intestinal epithelium by ethanol. Thereafter, Dr. Meena investigated the influence of ethanol on liver damage. Ethanol feeding increased the liver triglyceride level that was associated with histological lesions and elevated plasma AST/ALT in WT mice but not in *Trpv6*$^{-/-}$ mice. As in the colon, ethanol feeding increased liver cytokine mRNA expression (*Il-1β, Tnfα,* chemokine (C-X-C motif) ligand 1, chemokine (C-C motif) ligand 5) and reduced anti-oxidant mRNA expression (Thioredoxin1, superoxide dismutase 2, catalase) in WT mice, whereas these changes were blocked in *Trpv6*$^{-/-}$ mice. These data demonstrate that TRPV6 plays an important role in ethanol-induced colonic epithelial TJ disruption, mucosal barrier dysfunction, endotoxemia, and liver damage. This suggests that TRPV6 channel blockers may bear therapeutic value in the treatment of ALD and other alcohol-related diseases.

The following talk "Alcohol-induced adipocentric immunometabolic dysregulation", was presented by Dr. Flavia M. Souza-Smith, from the Louisiana State University Health Sciences Center. Alcohol consumption has significant immunomodulatory effects that impact innate and adaptive immune function, impairing inflammatory responses, and antimicrobial defense (Lau et al., 2009). The integrity of lymphatic vessels is crucial for antigen transport and immune cell regulatory functions (Brookes et al., 2009). Leaky lymphatic vessels allow antigens and immune cells, such as dendritic cells (DCs), to enter the perilymphatic adipose tissue (PLAT) (Fonseca et al., 2015). In the adipose tissue, DCs stimulate the expansion of fat regulatory T cells (fTregs) (Zou, Caton, Koretzky, & Kambayashi, 2010). Accumulation of fTregs in adipose tissue is a driver of age-associated insulin resistance (Bapat et al., 2015). Dr. Souza-Smith's previously published studies show that alcohol administration resulted in mesenteric lymphatic hyper-permeability; PLAT inflammatory milieu; increased PLAT CD4^{+} T cells, macrophages, and DCs; and impaired PLAT insulin signaling (Souza-Smith, Ford, Simon, & Molina, 2017; Souza-Smith, Siggins, & Molina, 2015). These findings were specific to the PLAT depot and not present in other adipose depots. Using flow cytometry, insulin-stimulated 2-deoxy-D-glucose uptake measurement, and DC migration assay, Dr. Souza-Smith investigated alcohol-induced lymphatic vessel/PLAT immune crosstalk. Male Fisher 344 rats received Lieber-DeCarli liquid diet that provided 36% of calories from alcohol for 10 weeks. Alcohol led to increased fTregs in PLAT, decreased Tregs in peripheral blood lymphocytes, and decreased CD4/CD8 ratio in mesenteric lymph nodes. Explants of PLAT from alcohol-treated animals had reduced glucose uptake and attracted more DCs than pair-fed controls. Together, these

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data suggest that alcohol-induced lymphatic leakage might promote DC entry into PLAT, leading to decreased mucosal immunity and immunometabolic dysregulation.

Dr. Philip Roper, a postdoctoral fellow in the laboratory of Dr. John J. Callaci from Loyola University of Chicago, presented the next talk titled "Ethanol-induced suppression of mesenchymal stem cell (MSC) osteochondrogenic differentiation is associated with increased forkhead box, class O signaling". Alcohol abuse is associated with an increased risk of fracture healing complications (Zura et al., 2016). Their laboratory has recapitulated these findings in various rodent models (Roper, Abbasnia, Vuchkovska, Natoli, & Callaci, 2016). Using a repeated binge model of alcohol exposure, they have shown that the formation of the cartilaginous callus is inhibited, and markers of endochondral ossification, such as hypertrophic chondrocyte accumulation, are reduced. Because fracture healing through endochondral ossification relies heavily upon proper MSC differentiation toward osteoblasts and chondrocytes, they hypothesized that alcohol exposure could be directly inhibiting MSC differentiation in the bone marrow and around the fracture callus, leading to the perturbed fracture healing. To test this, they harvested MSCs from the bone marrow of tibiae and femurs of male Lewis rats and cultured them *in vitro*. They exposed cells to growth medium, differentiation medium, or differentiation medium with 50 mM ethanol added. Total RNA was harvested from these cells after 48 and 72 h, and real-time PCR was used to analyze the relative change in expression of pro-osteoblastic or prochondrogenic genes. They found that the osteoblastic differentiation medium was able to induce the expression of runt-related transcription factor 2 and alkaline phosphatase at 48 h of exposure, while concomitant ethanol exposure suppressed the induction of these genes. At 72 h, the osteoblastic differentiation medium induced the expression of alkaline phosphatase, collagen alpha-1 type I, and osteocalcin, while ethanol suppressed the induction of these genes and decreased the expression of runt-related transcription factor 2. Next, they examined the effects of alcohol exposure on the expression of forkhead box, class O transcription factors in MSCs because these transcription factors can inhibit canonical Wnt signaling, which is necessary to drive MSC osteochondroblastic differentiation. Ethanol increased forkhead box, class O mRNA and protein within MSCs as early as after 3 h of treatment. Taken together, these findings begin to elucidate a possible mechanism by which alcohol could be mediating the deleterious effects seen on fracture healing.

Session 4: Health Consequences and Alcohol Drinking

The next session, "Health Consequences and Alcohol Drinking", was chaired by Dr. Matthias Majetschak (Loyola University Chicago) and Jordan Bowman (University of Nebraska Medical Center). They invited Dr. Michelle Foster from Colorado State University, Fort Collins to present her talk "High fat diet-induced alterations in lymph node structure and function". Obesity is characterized as a state of chronic low-grade inflammation proposed to be driven by unhealthy adipose tissue. She proposed that unhealthy visceral adipose tissue negatively influences immunity, by interactions with the lymphatic system. Lymph nodes are embedded in adipose tissue depots (Pond & Mattacks, 1995) and they act as filters and barriers against pathogen expansion. She hypothesized that tissue inflammation drains into lymph nodes, altering their structure and function. Previously, they demonstrated in a mouse model that adipose tissue inflammation induced by 8 weeks of a high fat diet

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(HFD) was associated with visceral lymph node hypertrophy driven by increased pro-inflammatory immune cells (Magnuson et al., 2017). In addition, they have shown that the gastrointestinal tract influences immune cells within the visceral lymph node (Magnuson et al., 2018). Thus, they postulate that the visceral lymph nodes play a primary role in the exacerbation of systemic inflammation linked to HFD-induced obesity. In the presented study, they examined how chronic HFD alters lymph node structure and function. They used male C57BL/6 mice fed chow or HFD for 13 weeks. At termination, the visceral and subcutaneous adipose depots and lymph nodes were collected. In contrast to 8 weeks of HFD, 13 weeks of HFD caused a significant decrease in the number of immune cells in visceral, but not subcutaneous, lymph nodes. This was associated with significant collagen deposition in central and sub-capsular areas of the visceral lymph node. Consistent with reductions of viable immune cell populations, mitogen stimulation of T cells collected from the visceral lymph nodes of HFD-fed mice had reduced proliferative capacity compared to those of the chow mice. This suggests that visceral adipose tissue inflammation drives lymph node fibrosis, leading to changes in its mechanical infrastructure necessary for immune cell priming. However, more studies are needed to confirm whether similar mechanisms exist in the setting of chronic alcohol use.

The next speaker was Dr. S. Vamsee Raju from the University of Alabama at Birmingham. His presentation was titled "Role of acquired cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction in alcohol impairment of mucus clearance". Delayed mucociliary clearance is considered to increase the risk of respiratory infections among excessive alcohol users (Sisson, 2007; Venizelos, Gerrity, & Yeates, 1981). Dr. Raju's group reported that nasal potential difference measurements in a Lieber-DeCarli model using male and female Sprague Dawley rats indicate that alcohol reduces the ion transport function of the CFTR, the dysfunctional protein in cystic fibrosis lung disease. In these rats, alcohol-induced reduction in CFTR function by 35% was associated with a 40% loss of mucociliary clearance as determined by radiographic clearance assay of inhaled Tc99 label *in vivo*. Consistent with CFTR dysfunction (Raju et al., 2017), micro-optical coherence tomography imaging of trachea excised from alcohol-treated rats exhibited decreased periciliary layer depth. Analysis of lung tissues from these rats indicated no change in expression of CFTR mRNA and protein, while displaying increased alcian blue periodic acid-Schiff mucus staining and mucin 5B. These expression data were supported by elevated mucin 5B protein in bronchial lavage from subjects with a history of alcohol use disorders per the Alcohol Use Disorders Identification Test. A subset of both alcohol and control rats were challenged with *Klebsiella pneumoniae*, a bacterial pathogen to which alcohol users are susceptible. Compared to controls, computed tomography imaging and histopathological examination of lung sections from alcohol-treated animals exhibited more evidence of multifocal pneumonia with edema, fibrin deposition, and numerous bacteria in alveolar spaces. In summary, Dr. Raju's findings suggest a role for CFTR dysfunction in compromised lung defense leading to an increased incidence of pneumonia among alcohol abusers.

Next, Dr. S. Alex Marshall from High Point University presented his work titled "Changes in cytokine and glial responses induced by binge-like drinking under nondependent conditions". Dependence on alcohol is associated with dysregulation of the neuroimmune system. This dysregulation has been associated with both a hyper- and hypo-reactive

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response depending on conditions (e.g., abstinence vs. intoxication, blood ethanol concentration levels, etc.) (Marshall, Geil, & Nixon, 2016). Maladaptations to the neuroimmune response are proposed as a potential mechanism of alcohol-induced brain damage. However, many individuals who participate in binge drinking show neither signs of dependence nor evidence of neurodegeneration. As such, it is critical to consider how binge drinking alone manipulates the neuroimmune response. Previous reports have focused on binge-like consumption effects on the neuroimmune system in the amygdala (Marshall et al., 2016; Marshall, McKnight, Blose, Lysle, & Thiele, 2017). The purpose of this study was to determine how components of the neuroimmune system in the hippocampus are modulated by binge drinking. Binge-like consumption was recapitulated using the Drinking-In-the-Dark procedure in male C57BL/6J mice, a well-established rodent model of alcohol abuse that does not lead to dependence or brain damage. Following the Drinking-In-the-Dark procedure, tissue slices were obtained from extracted mouse brains and subjected to immunohistochemistry to determine both cytokine and glial responses. Dr. Marshall's results indicated that binge-like ethanol consumption resulted in a 3.6-fold increase in IL-1β and a 67% decrease in IL-10 in various regions of the hippocampus, compared with the water-fed control group. Moreover, astrocyte activation occurred following ethanol exposure, as glial fibrillary acidic protein immunoreactivity was increased over 120%. Although initially one Drinking-In-the-Dark cycle had no effect on microglia number, three cycles reduced the number of microglia in various subregions of the hippocampus. However, the lack of effects in ionized calcium binding adaptor molecule 1 immunoreactivity led him to infer that alcohol may reduce the number of microglia but that those surviving may be more activated. Altogether, these findings suggest that binge-like ethanol drinking prior to dependence causes dysregulation to the neuroimmune system. This altered neuroimmune state may have an impact on behavior but could also result in a heightened neuroimmune response to subsequent immunomodulatory events and potentially exacerbate neurodegeneration. Understanding the impact of various types of ethanol exposure on glial responses and the persistence of these effects is critical to elucidate the neurobiological consequences of excessive consumption.

The final presentation of the session and meeting was by Dr. Mayumi Fujita from the University of Colorado Denver. Her talk was titled "A novel phenotype of Aldehyde dehydrogenase 2 (ALDH2) polymorphism: alcohol-induced skin pigmentation". Aldehyde dehydrogenase 2 is an enzyme that oxidizes carcinogenic acetaldehyde into non-toxic acetic acid (Marchitti, Brocker, Stagos, & Vasiliou, 2008). The mechanism of aldehyde toxicity lies in the highly reactive carbonyl group that can lead to the formation of aldehyde adducts on proteins, lipids, and nucleic acids (Marchitti et al., 2008; Vasiliou, Thompson, Smith, Fujita, & Chen, 2013). Counteracting the toxicity of aldehydes, ALDH enzymes promote oxidation of endogenous and exogenous aldehydes to carboxylic acid, ester hydrolysis of carboxylic and inorganic acids, and denitrification of organic nitrites. About 40% of East Asians have a defective polymorphism of *ALDH2, ALDH2*2,* resulting in the accumulation of acetaldehyde after drinking alcohol (Chen, Ferreira, Gross, & Mochly-Rosen, 2014). While studying the effect of the functional defect of ALDH2 using male and female *Aldh2* whole body knockout C57BL/6 mice, Dr. Fujita and her group unexpectedly found skin pigmentation in mice. The pigment was ethanol dose-dependent and reversible after

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discontinuation of ethanol administration. Histological analysis showed that the pigment was melanin and mainly localized in the epidermis. Human studies with 190 Japanese males (118 healthy volunteers and 72 alcoholics) confirmed the strong association between alcohol intake and skin pigmentation. The *ALDH2*2* polymorphism is a risk factor for esophageal melanosis caused by excessive alcohol intake and is correlated with esophageal cancerous diseases (Yokoyama et al., 2005). However, the direct link between ALDH2 and skin pigmentation has not been well studied. Dr. Fujita is currently studying mechanisms of melanin deposit and melanocyte biology induced by ethanol in *Aldh2* knockout mice. In summary, she reported an unexpected phenotype of a defective allele of the *ALDH2* gene, *ALDH2*2,* leading to ethanol-induced skin melanosis in a mouse model and in human subjects.

Summary

The 2018 AIRIG meeting covered a wide range of topics examining the effects of alcohol on the immune system, infection, and patient populations. The ability of alcohol to promote infection and delay activation of immune processes was highlighted by numerous speakers. In lung transplant patients and IBD patients, alcohol use was found to be associated with increased lung infections and intestinal infections, respectively. In animal models, alcohol potentiated the inflammatory response after *S. pneumoniae* infection in aged mice, while in rats, alcohol decreased CFTR function, coincident with the worsening of pneumonia-like symptoms after *K. pneumoniae* infection. During sepsis, alcohol delayed activation of the immune system and compromised gut integrity, while treatment with EGF had a protective effect. With regard to immunometabolic changes, speakers presented that DCs accumulate in PLAT after alcohol feeding, hepatocyte-specific deletion of TLR4 abrogates alcohol-induced liver injury and triglyceride accumulation, and the possibility that chronic alcohol intake can drive visceral adipogenesis. In liver and alcohol biology, speakers presented the utilization of precision-cut liver slices to characterize responses to alcohol in different strains of mice, the finding that alcohol induced hepatic stress leading to circulating mtDNA-enriched microparticles that heighten the inflammatory response, and the novel discovery that alcoholic hepatitis might be a result of bacterial infection. Alcohol binge mouse models were shown to have deleterious effects, like impairing fracture healing by limiting MSC differentiation, and increasing neuroimmune cell activation, possibly playing a role in neurodegeneration. After alcohol treatment, deletion of TRPV6 prevents cytokine induction and reactive oxygen species-induced damage in the intestine. In addition to alcohol's more internal effects, impaired alcohol metabolism due to ALDH2 knockout in mice or a defective allele in humans led to the increased skin pigmentation, the downstream effects of which are not known. Taken together, the data demonstrate the diverse systemic effects of alcohol, and underscore the importance of continuing research related to alcohol use.

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