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DUAL ROLE OF ANTIMICROBIAL PEPTIDE CATHELICIDIN IN
ALCOHOL-ASSOCIATED LIVER DISEASE

By
Fengyuan Li
M.S. University of Louisville, 2018

A Dissertation
Submitted to the Faculty of the
School of Medicine of the University of Louisville
In Partial Fulfillment of the Requirements
for the Degree of

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Department of Pharmacology and Toxicology
University of Louisville
Louisville, KY

May 2020

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Dissertation Approved on

April 16, 2020

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DEDICATION

This dissertation is dedicated to

My parents

For their endless love, support and encouragement.

And

My grandparents

For their belief and foresight in education.

And

Wenke Feng, Ph.D.

For his mentorship with wisdom, selflessness, and a dedication to education.

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First and foremost, I would like to thank my mentor, Dr. Wenke Feng, who have provided pivotal guidance and support to my development as a scientist. I am extremely grateful for the help and support from my committee members, Dr. Craig J. McClain, Dr. Leila Gobejishvili, Dr. Nobuyuki Matoba and Dr. Zhongbin Deng. Many thanks to the current and former members of Feng's lab, for helping me from the very beginning of my years in the States. I am also especially appreciative to individuals in the alcohol research center and the collaborators, for the guidance and support over the years on several major projects. Thanks to the department of Pharmacology and Toxicology and the IPIBS program for providing me the opportunity to study abroad and pursue my research in Dr. Feng's lab. Last but not the least, I would like to thank my beloved parents, sister and Mr. C.Y. Yong, who have been unconditionally supportive and encouraging throughout my time as a Ph.D. student at the University of Louisville.

ABSTRACT

DUAL ROLE OF ANTIMICROBIAL PEPTIDE CATHELICIDIN IN
ALCOHOL-ASSOCIATED LIVER DISEASE

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April 16, 2020

Patients with alcohol-associated liver disease (ALD) have high morbidity and mortality in its severe forms. One of the major features in different forms of ALD is the altered gut microbiota. Antimicrobial peptides (AMPs) play a crucial role in maintaining gut microbiota homeostasis. LL-37 (known as CRAMP in mouse) is the sole member of the human cathelicidin family and has piqued great research interest for its dual role in modulating microbiota and the immune response in metabolic diseases. Inflammasome activation is an important component of the liver pathophysiology in ALD and requires two signals for its full activation to induce the maturation and release of IL-1 β , to induce inflammation. Whether LL-37/CRAMP is involved in the regulation of inflammasome activation in ALD remains unclear. The first aim of this dissertation was to investigate whether CRAMP protects the liver from alcohol-induced injury and whether the effect is mediated through regulation of gut microbiota and inflammasome activation inhibition. Indeed, CRAMP deficiency exacerbated, while exogenous CRAMP peptide alleviated, alcohol-induced liver injury through inhibition of inflammasome activation by decreasing LPS function and uric acid (UA) production.

Obese alcoholic subjects have elevated serum ALT levels and are often at high risk of progressing from simple fatty liver to advanced ALD. Increased neutrophil infiltration and chemokine expression were linked to the synergistic effects of alcohol consumption and obesity. LL-37 and CRAMP has been recognized as chemoattractants to neutrophils and inflammatory monocytes through membrane chemokine receptors. The second aim was to investigate the role of CRAMP in binge alcohol-induced liver injury in high fat diet (HFD)-fed mice. CRAMP knockout (*Camp*^{-/-}) mice were protected from HFD feeding induced weight gain and adipocyte enlargement, liver injury and steatosis; importantly, *Camp*^{-/-} mice had significantly fewer infiltrated neutrophils in the liver and decreased chemokine expression compared to WT mice. High fat diet plus ethanol (HFD+E) treatment significantly increased hepatic protein levels of CRAMP, which co-localized with Ly6G-positive neutrophils.

In summary, the dual effects of CRAMP in alcohol-associated liver disease have been explored. CRAMP plays a protective role in binge alcohol-induced liver injury in chronic alcohol-fed mice, through the inhibition of LPS function and UA-induced inflammasome activation. In contrast, CRAMP acts as a chemoattractant for neutrophil infiltration in HFD-fed mice challenged with binge alcohol, exacerbating liver injury. The present study suggested that the role of CRAMP in alcohol-associated liver disease is dependent on the disease status and targeting CRAMP may represent a novel therapeutic strategy for alcohol-associated liver disease.

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CHAPTER 1 INTRODUCTION

1.1 Overview of Alcohol-associated Liver Disease (ALD)

The excessive and long-term consumption of alcohol causes serious health consequences to individuals and can also exert heavy burdens onto the society and economies worldwide. Accounting for 3.3 million deaths in 2012 ¹ and ranking as the fifth leading global cause of preventable morbidity ^{2,3}, the harmful use of alcohol over decades damages nearly every organ in the body, including the brain ⁴, heart ⁵, pancreas ⁶ and liver ⁷. Excessive alcohol drinking is strongly associated with several types of cancer including breast cancer ⁸ and colorectal cancer ⁹, and studies have shown that high doses of alcohol consumption can directly suppress a wide range of immune responses, and that alcohol abuse is associated with an increased incidence of a number of infectious diseases ¹⁰.

The liver acts as the major organ for alcohol metabolism, and thus it sustains the earliest and greatest degree of tissue injury from excessive alcohol drinking ^{11,12}. Alcohol-associated liver disease (ALD) is a term that encompasses the liver manifestations of alcohol overconsumption, including fatty liver, alcohol-associated hepatitis, and chronic hepatitis with liver fibrosis or cirrhosis, and sometimes progressing to liver cancer, or hepatocellular carcinoma (HCC) ¹³ (Figure 1.1). ALD has profound economic and health impact worldwide. According to the National Institute on Alcohol

Abuse and Alcoholism (NIAAA) Surveillance Report 2016, liver cirrhosis ranked as the 12th leading cause of death in the United States, and about half of all cirrhosis deaths in 2013 were alcohol related ³. Steatosis is the earliest and most common response in most heavy drinkers, and is usually reversible ¹⁴. Alcohol-associated hepatitis is a more severe, inflammatory type of liver injury characterized by swollen, dying hepatocytes (i.e., ballooning degeneration), infiltrated neutrophils and inflammatory monocytes, and the development of tangled aggregates of insoluble proteins called Mallory-Denk bodies within hepatocytes. The activation of Kupffer cells (KCs), the resident macrophages in the liver, is critical in alcohol-associated hepatitis development ¹⁵. Fibrosis and its terminal or late stage, cirrhosis, refer to the deposition of abnormal amounts of extracellular matrix proteins, principally by activated hepatic stellate cells (HSCs). Patients initially exhibit active pericellular fibrosis, which may progress to cirrhosis, the late stage of hepatic scarring ¹⁶.

Modified from Gao and Bataller, Gastroenterology, 2011

Figure 1.1 Progression of alcoholic liver disease (ALD).

Classic treatment of ALD includes abstinence, which is the most effective way to manage ALD for subjects at early disease stage ¹⁷. Nutritional support, corticosteroids, and liver transplantation are also used based on disease severity and other complications ¹⁸⁻²⁰. Despite major progress made in the past decades in understanding the pathogenesis of ALD and the improvement in ALD management, currently there is still no FDA (Food and Drug Administration) approved therapy for any stage of ALD. New targeted therapies for ALD are urgently needed.

It is important to understand how ethanol damages the liver. Ethanol is mainly metabolized in hepatocytes, the main parenchymal cells of the liver, which make up to about 70 percent of the liver mass ²¹. Hepatocytes express the highest levels of two major enzymes involved in ethanol metabolism, alcohol dehydrogenase (ADH) in the cytosol and cytochrome P450 2E1 (CYP2E1), which resides in the smooth endoplasmic reticulum (ER). Ethanol is oxidized to acetaldehyde by ADH and then to acetate in the mitochondria by aldehyde dehydrogenase 2 (ALDH2). These sequential oxidation processes produce two mole equivalents of reduced nicotinamide adenine dinucleotide (NADH), which can alter the cellular redox potential and can enhance lipid synthesis. The oxidation of ethanol can also take place in the presence of molecular oxygen and hydrogen peroxide by CYP2E1 and another enzyme that is highly expressed in hepatocyte, catalase. These two pathways also generate acetaldehyde, contributing to further oxidation of acetaldehyde to acetate by ALDH2 in the mitochondria ²². Acetaldehyde is a highly toxic substance and a known carcinogen ²², while acetate is less active and can be broken down into water and carbon dioxide for easy elimination. Acetaldehyde can covalently bind to proteins, lipids and nucleic acids to form

acetaldehyde adducts, which can, in turn, disrupt the structure and function of these macromolecules ^{11,23}. This is particularly evident in the liver, where the bulk of alcohol metabolism takes place. Some alcohol metabolism also occurs in other tissues, including the pancreas ²⁴ and the brain, causing damage to cells and tissues. It has been shown that small amounts of alcohol are metabolized to acetaldehyde in the gastrointestinal tract, exposing these tissues to acetaldehyde's damaging effects ²⁵. Additionally, small amounts of alcohol are also removed by interacting with fatty acids to form compounds called fatty acid ethyl esters (FAEEs). These compounds have been shown to contribute to damage to the liver and pancreas ²⁴.

The oxidative pathway of alcohol metabolism generates the toxic metabolite, acetaldehyde, and it gives rise to large amounts of reactive oxygen species (ROS), which are considered the key toxins in alcohol-mediated liver injury ²⁶⁻³⁰. In heavy drinkers, accelerated alcohol metabolism by higher levels of CYP2E1 puts liver cells in metabolic peril, because more CYP2E1 not only produces more acetaldehyde, but also generates greater amounts of various other ROS, including hydroxyethyl radicals (i.e., free-radical forms of ethanol), superoxide anions (O_2^-) and hydroxyl radicals ($\cdot OH$). Continuous generation of these reactive molecules eventually creates the condition known as oxidant stress or oxidative stress. Under these conditions, the rate of ROS generation exceeds the liver's capacity to neutralize them with natural antioxidants, such as glutathione and vitamins E, A, and C, or to remove them using antioxidant enzymes. Animal studies showed that chronic ethanol consumption leads to decreased activities and/or amounts of several antioxidant enzymes, which worsens the hepatocytes' oxidant burden ³¹⁻³³.

Oxidant stress is further exacerbated when the generated ROS undergo secondary reactions with proteins and unsaturated lipids ¹¹.

Impaired innate and adaptive immunity is another hallmark of ALD. Alcohol can impair both innate and adaptive immunity, either directly through ROS production or indirectly by increasing gut permeability and consequently increasing endotoxin and bacterial translocation that contribute to the development of ALD ³⁴. On one hand, the metabolism of alcohol itself leads to the production of ROS, and the formation of immunogenic protein adducts (which induces an immune reaction with antibody production or T-cell activation, or both), causing DNA damage, mutagenesis and direct cell death, contributing to tissue damage. On the other hand, alcohol consumption causes leaky gut and reduction of antimicrobial peptides (AMPs), which allows the translocation of pathogen-associated molecular patterns (PAMPs) such as LPS into the portal circulation. The activation of KCs by LPS results in up-regulation of pro-inflammatory cytokines through the activation of nuclear factor kappa B (NF- κ B), which promotes the development of ALD. Several components of the innate and adaptive immune responses are involved in the hepatic response to injury. Within the liver, PAMPs are presented to Toll-like receptors (TLRs) on macrophages and induces the expression of inflammatory cytokines and chemokines, causing liver injury, for example, interleukin (IL)-1 β , tumor necrosis factor (TNF)- α , chemokine (C-X-C motif) ligand (CXCL)-1 and CXCL-2. CXCL-1 and CXCL-2 act as chemoattractants through the chemokine receptor C-X-C motif chemokine receptor (CXCR)-1 and CXCR-2 on neutrophil to recruit neutrophils, which induces more pro-inflammatory cytokine expression and further causing liver injury. T helper 17 (Th17) cells are activated and secret pro-inflammatory cytokine IL-17

which further increase neutrophil infiltration. In addition, TNF- α and fas cell surface death receptor (Fas) produced by T cells also leads to apoptotic hepatocyte cell death through the Fas death receptor and TNF receptor pathways ³⁵.

Obesity has long been recognized as one of the most common comorbidities of ALD, and it can strongly affect the initiation and progression of the disease. The interaction of excessive alcohol drinking and obesity has been confirmed in a large number of comprehensive clinical studies involving obese alcoholics, non-obese alcoholics and obese non-alcoholics as controls. Higher serum ALT levels were often found in obese alcoholics and this group of alcoholic subjects usually has a higher risk of progressing from fatty liver to advanced forms of ALD ³⁶⁻⁴³. Oxidative stress and nitrosative damage were linked to the synergistic effects of obesity and alcohol consumption in diet-induced obesity animal models. An increased pro-inflammatory response and impaired hepatic lipid metabolism were seen in genetically obese rodent models ⁴⁴. A recent study showed that feeding mice with short- or long-term high fat diet (HFD) plus a single ethanol binge synergistically increased the serum and hepatic free fatty acids, chemokine CXCL1 expression and exaggerated neutrophil infiltration in the liver, eventually leading to severe liver injury ⁴⁵.

1.2 The Gut-Liver Axis

Gut microbiota changes have been identified as one of the major features in different stages of ALD, emphasizing the importance of gut-liver axis relevant to ALD ^{46,47} (Figure 1.2). Although alcohol is mainly metabolized in the liver, it is well-known that alcohol consumption causes gut lumen bacterial overgrowth and dysbiosis, intestinal

mucosal damage, and increased intestinal permeability, leading to increased translocation of bacteria and their products, such as endotoxin (mainly lipopolysaccharide, LPS) into the portal circulation and binds with LPS binding protein (LBP). Bacteria and their products stimulate the production of ROS and pro-inflammatory cytokines and chemokines, resulting in damage to liver cells and the development of liver injury^{48,49}. Gut bacteria-derived endotoxin acts through pattern recognition receptors such as toll-like receptors (TLRs) which are expressed in liver resident macrophages--KCs--as well as other cell types in the liver. The major endotoxin, LPS, is derived from the cell walls of gram-negative bacteria in the gut lumen, binds with LBP and recognizes TLR4 and its co-receptors, CD14 and MD2 in the liver when penetrating the intestinal barrier and entering into blood stream. Deficiency in the TLR4 complex, such as mutation of TLR4, lack of CD14 and/or MD2, protects mice from alcohol-induced liver injury. It has been widely demonstrated that alcohol consumption induces endotoxemia^{50,51}. These observations suggest that gut bacteria homeostasis, intestinal barrier integrity, and hepatic TLRs are important in the pathogenesis of ALD.

Modified from Tripathi et. al, Nature Reviews Gastroenterology & Hepatology, 2018

Figure 1.2 The gut-liver axis.

Accumulating evidence demonstrated that intestinal dysbiosis is a risk factor in ALD development/progression. Intestinal dysbiosis is defined as an imbalance of the different microbial entities in the intestine with a disruption of symbiosis ⁵². Both chronic and acute alcohol consumption lead to bacterial overgrowth and dysbiosis in both the small and large intestine in experimental animals ^{29,52-54} and in human alcoholic subjects ⁵⁵⁻⁶⁰.

In a rat model of ALD using intragastric gavage feeding, Multu and colleagues showed that alcohol ingestion did not change microbiota composition in 4-6 weeks of feeding, but did significantly alter the mucosa-associated microbiota in the colon after 10-weeks of feeding ⁵⁰. A recent study by Yan et al. further demonstrated that 3-weeks of alcohol ingestion in mice led to bacterial overgrowth in the proximal small intestine, and dysbiosis, which was associated with the suppression of the anti-microbial peptides, Reg3 β and Reg3 γ . Moreover, the population of *Lactobacilli* was depleted in alcohol-fed mice ⁶¹. Germ-free mice had less fat in the liver after alcohol feeding compared to conventional mice; moreover, transplantation of intestinal contents from conventional mice to germ-free mice induced inflammation in both intestine and liver ⁵⁴.

In 1984, Bode and co-workers found that the bacterial population was increased in the jejunum of alcoholics compared with hospitalized control patients ⁶². These same investigators further showed a higher prevalence of small intestine bacterial overgrowth in chronic alcoholics compared to controls using a breath test ⁶³. These observations were later confirmed by other groups ^{57,59}. Recently, Bajaj et al. studied bacterial composition in the 244 alcoholic cirrhotic patients and 25 age-matched controls. Using an index, cirrhosis dysbiosis ratio (CDR, a low number indicating dysbiosis), the authors found that

intestinal dysbiosis was more severe in decompensated cirrhotics compared to compensated cirrhotics ⁶⁴. Using the lactulose breath test, Gabbard and co-workers observed that moderate drinking was a strong risk factor for small intestine bacterial overgrowth ⁶⁵.

1.3 Hepatic Inflammation and Inflammasome Activation

Inflammatory reactions play an important role in the pathogenesis of ALD. Inflammatory processes are primary contributors to the development and progression of alcohol associated steatohepatitis (ASH), with severe AH characterized by non-resolving inflammation ⁶⁶. Hepatic inflammation in the development and progression of ASH is a complex response to gut dysbiosis, impaired intestinal barrier function, hepatocellular stress and death, as well as inter-organ crosstalk. In response to chronic, excessive alcohol exposure, hepatocytes themselves also contribute to the inflammatory process. The hepatocytes produce large number of chemokines and inflammatory mediators and release damage-associated molecular patterns during injury and death. These cellular responses are mediated and accompanied by changes in the expression of pro- and anti-inflammatory cytokines and chemokines, as well as by signals which orchestrate the recruitment of immune cells and activation of the inflammatory process ⁶⁷.

The innate immune system represents the first-line response to invading microbes and tissue damage. Many of the proteins and cells involved in innate immunity are produced by, and reside in, the liver. This abundance in immune cells and proteins reflects the liver's adaptation to various immune challenges but also makes the organ particularly vulnerable to alcohol's effects ⁶⁸. Heavy alcohol consumption exacerbates the

translocation of microbes and microbial products from the gut lumen to the liver via the portal vein. Alcohol and its toxic metabolites further damage the gut barrier. These immune challenges disrupt the liver's normally fine-tuned immune signaling pathways, leading to activation of various pathogen- or damage-associated molecular patterns, which induce the expression of pro-inflammatory cytokines results in cellular dysfunction that contributes to ALD.

Multiple interventions targeting the innate immune system have emerged in treating ALD. These treatments could include antibodies against pro-inflammatory cytokines, use of anti-inflammatory cytokines, or suppression of alcohol-induced epigenetic regulators of innate immunity. Stimulation of innate immunity is increasingly recognized to play an important role in the pathogenesis of ALD, while the contribution of adaptive immunity has received less attention ⁶⁹.

Inflammasome activation is an important component of the liver pathophysiology in ALD ^{70,71}. The NLRP3 inflammasome (an intracellular protein complex consisting of NLRP3, apoptosis-associated speck protein with caspase activation and recruitment domain (ASC), and caspase-1) is one of the most studied inflammasomes. The NLRP3 inflammasome requires both Signal 1 (Pathogen-associated molecular patterns, PAMPs) and Signal 2 (Danger-associated molecular patterns, DAMPs) for its full activation. The activation of caspase-1 enables the cleavage of pro-interleukin (IL)-1 β to form active IL-1 β , which can then be secreted and bind to the IL-1 receptor to induce inflammation ⁷².

1.4 Antimicrobial Peptides, Cathelicidin, and Their Roles in Liver Diseases

One of most important mechanisms maintaining gut microbiota homeostasis is antimicrobial peptides (AMPs)-mediated regulation. Intestinal goblet cells-produced mucin forms the mucus layer, serving as the first defense against bacterial invasion into the mucosa. AMPs are produced in Paneth cells, enterocytes, intestinal epithelial cells and macrophages. They form a central part of the innate immune system to counter microbial infections ⁷³ and inhibit the overgrowth of pathogenic bacteria in gut lumen ^{74,75}, serving as intrinsic regulators of gut eubiosis.

Previous studies have demonstrated the critical role of AMPs in the pathogenesis of ALD ^{61,76,77}. Lack of intestinal epithelial cell-derived Reg3 β or Reg3 γ (regenerating islet-derived (REG)-3 lectins) exacerbated alcohol-induced dysbiosis and ALD, while overexpression of *Reg3g* attenuated ALD ⁷⁶. The Paneth cell-produced antimicrobial peptide family, α -defensins, is another important group of antimicrobial peptides. Although expression of α -defensin 5 did not differ significantly in mice fed alcohol intragastrically for 3 weeks ⁶¹, dysfunction of α -defensin 5 exacerbated alcohol-induced luminal and mucus-associated microbiota alterations and resulted in a severe liver injury, while zinc supplementation increased α -defensin 5 production and reduced ALD ⁷⁸.

Alcohol consumption also decreased other types of antimicrobial peptides. Our previous studies showed a decreased expression of intestinal CRAMP (Cathelicidin-associated antimicrobial peptide) in mouse models of ALD, and that treatment with probiotics improved gut microbiota homeostasis and restored intestinal CRAMP expression in mice ^{79,80}.

CRAMP is a mouse ortholog of LL-37, the only member of the human cathelicidin family ⁸¹ (Figure 1.3). LL-37/CRAMP has piqued great research interest for the dual role in modulating microbiota and immune response ⁸² (Figure 1.4) in several infectious disease and inflammatory bowel disease (IBD) ⁸³⁻⁸⁵. Wild-type (WT) mice cohoused with *Camp*^{-/-} (CRAMP knockout) mice had markedly increased sensitivity to dextran sulfate sodium (DSS)-induced colitis, with a skewed composition of fecal microbiota ⁸⁶. Previous studies showed that cathelicidins also serve as immune modulators in metabolic diseases. CRAMP produced by β -cells in the pancreatic islets induces regulatory macrophages and DCs (dendritic cells), generating the pancreatic regulatory T cells; whereas in the absence or deficiency of CRAMP, the macrophages and dendritic cells exhibit an inflammatory profile, thus generating diabetogenic T cells, which attack the pancreas and destroy the insulin-secreting cells, leading to decreased insulin production and type1 diabetes ⁸⁷. Notably, the production of CRAMP in the pancreas is controlled by gut derived short chain fatty acids (SCFAs), which demonstrates the importance of gut microbiota in CRAMP regulation ⁸⁷. More recently, studies have shown that CRAMP suppresses hepatic steatosis in high fat diet-treated mice through an ERK- and CD36-dependent mechanism ⁸⁸.

Kao et al., mBio, 2016

Figure 1.3 The cathelicidin family.

Modified from Hancock & Sahl, Nat Biotechnol, 2006

Figure 1.4 Dual role of cathelicidin.

Inflammasome activation is an important component of the liver pathophysiology in ALD ^{70,71}. The NLRP3 inflammasome (an intracellular protein complex consisting of NLRP3, apoptosis-associated speck protein with caspase activation and recruitment domain (ASC), and caspase-1) is one of the most studied inflammasomes. The NLRP3 inflammasome requires both Signal 1 (Pathogen-associated molecular patterns, PAMPs) and Signal 2 (Danger-associated molecular patterns, DAMPs) for its full activation. The activation of caspase-1 enables the cleavage of pro-interleukin (IL)-1 β to form active IL-1 β , which can then be secreted out and bind to the IL-1 receptor to induce inflammation ⁷². LL-37 prevented the TLR-4 mediated pro-inflammatory signaling by inhibiting the binding of LPS to the CD14/TLR-4 complex and by promoting LPS degradation by lysosomes ⁸⁹⁻⁹¹. However, whether LL-37/CRAMP is involved in the regulation of inflammasome activation in ALD remains unclear.

Alcohol consumption and obesity work synergistically to promote ALD. A recent study showed that feeding mice with short- or long-term HFD plus a single ethanol binge synergistically increased the serum and hepatic free fatty acid levels, chemokine CXCL-1 expression and exaggerated neutrophil infiltration in the liver, eventually leading to severe liver injury ⁴⁵. Human cathelicidin LL-37 and mouse CRAMP have been demonstrated to act as a chemoattractant to neutrophils and inflammatory monocytes, through the chemokine receptor, CXCR2 ^{92,93}. This suggests a potential role of CRAMP in regulating neutrophil infiltration and chemokine expression in alcohol-induced liver injury in HFD-fed mice.

1.5 Specific Aims & Significance

Chronic liver disease either caused from alcohol abuse and high fat/high fructose diet place a heavy burden worldwide economically and sociologically. Although almost 95% of heavy drinkers develop the fatty liver, simple steatosis, only up to 40% of this population develops more severe forms of ALD. Several risk factors, including gut microbiota changes, contribute to this disease progression. AMPs that are predominantly expressed in the enterogastric tract play an important role in regulating bacteria growth and the translocation of bacteria and their products, mainly LPS. AMPs such as cathelicidin, LL-37 in human and CRAMP in mouse have piqued great research interest regarding their dual role in gut microbiota regulation as well as immune modulation. There were two specific aims in this dissertation. The first aim of this dissertation was to investigate whether CRAMP protects the liver from alcohol-induced injury and whether the effect is mediated through regulation of gut microbiota and inflammasome activation inhibition. The second aim was to investigate the role of CRAMP, a chemoattractant for neutrophil infiltration, in binge alcohol-induced liver injury in HFD-fed mice.

This dissertation aimed to investigate the role of CRAMP/LL-37 in alcohol-related liver disease in different animal models. The preconditioned fatty liver and impaired intestinal barrier function is of high clinical relevance as a large portion of patients with chronic liver disease also develop diabetes, obesity and have impaired gut barrier function. The unique properties of CRAMP in terms of the expression (CRAMP is expressed in multiple organ and diverse cell types) and function (antimicrobial activity, immune modulator and chemoattractant) gives great novelty and value to the present study in providing a new target in the treatment of alcohol-associated liver disease.

CHAPTER 2 ANTIMICROBIAL PEPTIDE CATHELICIDIN ALLEVIATES ALCOHOLIC LIVER DISEASE THROUGH INHIBITING LPS AND URIC ACID- INDUCED INFLAMMASOME ACTIVATION IN MICE

2.1 Introduction

Patients with alcohol associated liver disease (ALD) have high morbidity and mortality in its severe forms ^{13,94}. Altered gut microbiota has been identified as one of the major features in different forms of ALD ⁴⁶. Alcohol consumption causes dysbiosis and “leaky gut”, which allows the translocation of bacteria and their products through the portal vein into the liver, resulting in damage to the liver cells and the development of hepatic steatosis and liver injury through the toll-like receptor 4 (TLR4)-mediated pathway ^{48,49}.

One of the most important mechanisms maintaining gut microbiota homeostasis is antimicrobial peptides (AMPs)-mediated regulation. AMPs are produced in Paneth cells, enterocytes, intestinal epithelial cells and macrophages, and form a central part of the innate immune system to counter microbial infections ⁷³ and inhibit the overgrowth of pathogenic bacteria in the gut lumen ^{74,75}. Previous studies have demonstrated the critical role of AMPs in the pathogenesis of ALD ^{61,76,77}. Lack of intestinal epithelial cell-derived regenerating islet-derived (REG)-3 lectins, Reg3 β or Reg3 γ , exacerbated alcohol-induced dysbiosis and ALD, while overexpression of *Reg3g* attenuated ALD ⁷⁶. Recent work

further showed that alcohol-induced α -defensin dysfunction exaggerated endotoxin translocation and liver damage ⁷⁸. In addition to Reg3 lectins and defensins, cathelicidins are a major AMPs family. LL-37 is the sole member of the human cathelicidin family, and has a murine ortholog, cathelicidin-related antimicrobial peptide (CRAMP). LL-37/CRAMP has stimulated great research interest because of its dual role in modulating microbiota and immune responses in several infectious diseases and in inflammatory bowel disease (IBD) ⁸³⁻⁸⁵. Wild-type (WT) mice cohoused with *Camp*^{-/-} (CRAMP knockout) mice had markedly increased sensitivity to dextran sulfate sodium (DSS)-induced colitis, with a skewed composition of fecal microbiota ⁸⁶. CRAMP deficiency exacerbated type 1 diabetes through the dysregulation of β -cell immunity ⁸⁷. Notably, the production of CRAMP in the pancreas is controlled by gut derived butyric acid, implying the importance of gut microbiota in CRAMP regulation ⁸⁷.

Inflammasome activation is an important component of the liver pathophysiology in ALD ^{70,71}. The NLRP3 inflammasome (an intracellular protein complex consisting of NLRP3, apoptosis-associated speck protein with caspase activation and recruitment domain (ASC), and caspase-1) is one of the most studied inflammasomes. The NLRP3 inflammasome requires both Signal 1 (Pathogen-associated molecular patterns, PAMPs) and Signal 2 (Danger-associated molecular patterns, DAMPs) for its full activation. The activation of caspase-1 enables the cleavage of pro-interleukin (IL)-1 β to form active IL-1 β , which can then be secreted and bind to the IL-1 receptor to induce inflammation ⁷². LL-37 prevented the TLR-4 mediated pro-inflammatory pathway by inhibiting the binding of LPS to the CD14/TLR-4 complex and by promoting the LPS degradation by

lysosomes⁸⁹⁻⁹¹. However, whether LL-37/CRAMP is involved in the regulation of inflammasome activation in ALD remains unclear.

Our previous studies showed a decreased expression of intestinal CRAMP in mouse models of ALD, and the treatment with probiotics improved gut microbiota homeostasis and restored intestinal CRAMP expression in mice^{79,80}. In the current study, the hypothesis that CRAMP protects the liver from alcohol-induced injury through the regulation of inflammasome activation was tested. CRAMP deficiency exacerbates, while exogenous CRAMP peptide alleviates, alcohol-induced liver injury through inhibition of inflammasome activation by decreasing LPS function and uric acid (UA) production.

2.2 Materials and Methods

2.2.1 Animals

Camp^{-/-} mice were purchased from Jackson laboratory (#017799) and breed to obtain sufficient colonies for experiment. *Camp*^{-/-} male mice at the age of 8-10 weeks were used with their age-matched littermates WT mice (C57BL/6). Mice were maintained at 22°C with a 12h light/dark cycle and had free access to a normal chow diet and water. Mice were fed the Lieber DeCarli diet containing 5% alcohol (w/v) (Alcohol-fed, AF) or isocaloric maltose dextrin (Pair-fed, PF). For the AF groups, mice were initially fed the control Lieber-DeCarli liquid diet (Bio-Serve, Flemington, NJ) for 5 days to acclimate the mice to the liquid diet. The content of alcohol in the liquid diet of the AF groups was gradually increased from 1.6% (w/v) to 5% (w/v) in the first 6 days and remained at 5% for the subsequent 24 days. Mice in PF group were fed isocaloric Maltose Dextrin substituted for alcohol in the liquid diet for 24 days. To account for the

reduced food consumption of ethanol-fed mice, pair-fed mice were given the volume of diet consumed by their ethanol-fed counterparts in the previous day. On Day 24, 6 hours before harvesting, a bolus of EtOH (5 g/kg body weight) was given to alcohol-fed mice by gavage, while mice in PF groups received a gavage of maltose dextrin. The 38-amino acid CRAMP peptide (H-ISRLAGLLRKGGEKIGEKLLKKIGQKIKNFFQKLVPQPE-OH) was synthesized (Peptides International, Louisville, KY) and 100 µg CRAMP peptide in 100 µl saline was injected to alcohol-fed mice intraperitoneally (I.P.) once daily for 3 days prior to the conclusion of the feeding period. Serum and tissue samples were collected for analysis. All mice were treated according to the protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville.

2.2.2 Human Study

2.2.2.1 Alcohol-associated Hepatitis Study

Forty-seven male and female acute alcohol-associated hepatitis (AAH) patients (31 severe patients with Model for End-Stage Liver Disease [MELD] score ≥ 20 ; and 16 moderate patients with MELD score < 20) plus 11 healthy volunteers were included in this clinical study. This investigation was conducted as one of the aims of a large national multisite clinical trial (clinicaltrials.gov: NCT01809132) sponsored by the National Institute on Alcohol Abuse and Alcoholism (NIAAA). Patients were 21 to 66 years of age. Healthy controls (HCs) were of similar age and did not have liver disease or any other relevant disease conditions (heart, kidney, lung, neurologic or psychiatric illness, sepsis), and none of them showed any signs of a pro-inflammatory state⁹⁵.

2.2.2.2 AUD Study

Plasma samples from forty-eight male and female heavy drinking alcohol use disorder (AUD) patients from NIAAA were analyzed. Patients were grouped by serum alanine aminotransferase (ALT) levels (as a marker of liver injury) as AUD without ALD ($ALT \leq 40$ U/L, 7M/8F) and AUD with ALD ($ALT > 41$ U/L, 27M/6F). Demographics, drinking history (using lifetime drinking history [LTDH], and timeline follow back [TLFB] for the past 90 days) assessments, and serum uric acid were evaluated. All clinical laboratory assays were performed by the Department of Laboratory Medicine at NIH Bethesda MD per its guideline. Plasma IL-1 β levels were determined by multianalyte chemiluminescent detection using Multiplex kits (Millipore, Billerica, MA) on the Luminex (Luminex, Austin, TX) platform based on the manufacturers' instructions.

2.2.2.3 Liver Explants

Nine liver tissues (explants) from patients with severe AH were obtained from the Resource Center at Johns Hopkins University (n = 6 active drinkers, mean age 44 ± 10.1 , 2 female and 4 male patients) and University of Louisville Hospital (n = 3, mean age 57 ± 18.9 , one female and two male patients who were abstinent for more than 6 months). Five non-AH liver samples were also acquired from the Resource Center at Johns Hopkins University ⁹⁶.

All studies were approved by the respective Institutional Review Boards and written consent was obtained from all participants. All subjects consented to having their samples used in further research studies.

2.2.3 Cell Culture

2.2.3.1 Macrophage

RAW264.7 (mouse macrophage cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and were maintained at 37 °C and 5% CO₂. Bone marrow-derived cells were isolated from WT and *Camp*^{-/-} mice as previously described⁹⁷. Isolated cells were then cultured in modified DMEM medium with macrophage colony-stimulating factor (M-CSF), which is a cytokine that directs cell differentiation into macrophages. The bone marrow-derived macrophages (BMDM) were exposed to varying doses of synthetic CRAMP peptide for 2 hours prior to LPS stimulation (*Escherichia coli* 0111: B4, Sigma-Aldrich, St. Louis, MO) at 100 ng/ml for 4 hours. For UA-induced signal 2 transduction in inflammasome activation, RAW264.7 cells and BMDMs were primed with LPS at 500 ng/ml for 4 hours, then LPS was washed out with phosphate buffered saline (PBS), before treating the cells with UA (Uric acid, Sigma, St. Louis, MO) at 500 µM and CRAMP peptide for 4.5 hours in FBS-free DMEM culture medium. The CRAMP peptide was used at 1 µg/ml or 10 µg/ml in macrophage cell culture. Cell viability was not affected by LPS or CRAMP treatment at the doses used in the experiments. For ATP and LPS-induced inflammasome activation in RAW264.7 cells, ATP (Adenosine 5-triphosphate, Sigma, St. Louis, MO) was added at the concentration of 50 mM and cells were cultured for additional 30 mins after being primed with LPS (100 ng/ml) for 4 hours.

2.2.3.2 Hepatocyte and siRNA Transfection

Hepa1-6 (mouse hepatoma cell line) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and were maintained at 37 °C and 5% CO₂. Desferrioxamine (DFO) (Sigma, St. Louis, MO) at a concentration of 200 µM was used to treat the cells for 20 hours. For the ethanol treatment study, Hepa1-6 cells were treated with 400 mM ethanol (Decon Labs, King of Prussia, PA) for 4 hours. Knockdown of *Camp* in Hepa1-6 cells was achieved by siRNA transfection according to the manufacturer's protocol (Horizon, Waterbeach, United Kingdom). The siRNA targeting *Camp* (siRNA-*Camp*) (siGENOME Mouse Camp siRNA, SMARTPool, 5 nmol) and non-targeting control siRNA (siRNA-NT) (siGENOME Non-Targeting siRNA Control Pool #2) were suspended in siRNA buffer (5X siRNA buffer, Horizon) to the desired concentration. Hepa1-6 cells were seeded in 48-well plates the day before transfection. On transfecting day, siRNA and transfection reagent Lipofectamine (Lipofectamine™ RNAiMAX Transfection Reagent, ThermoFisher, Waltham, MA) were prepared separately in reduced serum medium (Opti-MEM Reduced Serum Medium, ThermoFisher, Waltham, MA) and mixed followed by an incubation at room temperature for 20 mins. The transfection medium was then added into the wells after removal of culture medium. Cells were continued in the transfection medium for 16 hours and the transfection medium was replaced with complete medium for an additional 8 hours before treatment.

2.2.4 ALT and AST Measurements

Serum activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using standard kits (Thermo Scientific, Waltham, MA).

2.2.5 Histological Analysis

2.2.5.1 Paraffin Embedded Liver Tissue

Liver tissues were collected and fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin-embedded tissue blocks were then sliced at 5 μ m thickness on a microtome and floated in a 40°C water bath containing distilled water. Sections were then transferred onto glass slides. Slides were dried overnight and stored at room temperature until ready for use. Before staining, slides were deparaffinized with Citrisolv (Decon, King of Prussia, PA) and rehydrated by immersing in graded ethanol solutions.

H&E Staining Hematoxylin and eosin (H&E) were used to stain the nuclear and cytoplasm components, respectively. After staining, sections were then dehydrated through graded alcohol, cleared in Citrisolv and then mounted with Cytoseal Xyl (Thermo Scientific, Waltham, MA) and observed by microscopy.

CAE Staining Infiltration of neutrophils in the liver was assessed by chloroacetate esterase (CAE) staining according to manufacturer's instructions (Naphthol AS-d Chloroacetate Kit, Sigma, St. Louis, MO). Briefly, liver sections were incubated in a solution of naphthol AS-D chloroacetate. The naphthol AS-D chloroacetate is enzymatically hydrolyzed by "specific esterase" in neutrophils, liberating a free naphthol

compound. This then couples with a diazonium compound, forming highly colored deposits at sites of enzyme activity that can be visualized under microscopy.

TUNEL Staining Apoptosis was detected via terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) staining according to the manufacturer's instructions (Millipore, Temecula, MA). In brief, the apoptotic cells *in situ* were identified by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the free 3'-OH of cleaved DNA. The biotin-labeled cleavage sites were then visualized by reaction with fluorescein conjugated avidin (avidin-FITC).

F4/80 Immunohistochemistry Following deparaffinization, antigen retrieval was performed on slides in 10mM citrate buffer (pH6.0) for 20 min at 95°C. Blocking buffer containing 1% bovine serum albumin (BSA) (Sigma, St. Louis, MO) was added onto the slides and the slides were incubated in a humidified chamber at room temperature for 1 hour, for the blocking of non-specific antibody binding. Sections were then incubated with primary antibody F4/80 (Abcam, Cambridge, UK) and AlexaFluor-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). DAPI (Invitrogen, Carlsbad, CA) was used for nuclei counterstain.

2.2.5.2 Frozen Liver Tissue

Liver tissues were immersed with optimal cutting temperature compound (OCT) and stored frozen in liquid nitrogen immediately after harvesting.

Oil Red O Staining Frozen sections were cut at 8µm to the slides and air dried. The slides were rinsed with 60% isopropanol and then stained with freshly prepared Oil Red O working solution for 15mins and rinsed again with 60% isopropanol. Nuclei were lightly

stain using haematoxylin for 45 seconds followed with 2 times rinse with distilled water. Slides were then mounted in aqueous mount and observed by microscopy.

DHE Staining Reactive oxygen species (ROS) accumulation in the liver was examined by dihydroethidium (DHE) staining. In brief, cryostat sections of liver were incubated with 5 μ mol/L DHE (Molecular Probes, Eugene, OR) for 30 min at 37°C in the dark.

Nonfluorescent dihydroethidium is oxidized by ROS to yield the red fluorescent product, ethidium, which binds to nucleic acids and stains the nucleus with bright fluorescent red. The red fluorescence was examined under confocal microscopy and the intensity of fluorescence was quantified using Image J.

2.2.6 Lipid Accumulation

Liver tissues were homogenized in 50 mM NaCl (Sigma, St. Louis, MO) solution in distilled water. The homogenates were then used for lipid extraction using methanol (Fisher chemical, Fair Lawn, NJ) and chloroform (Fisher chemical, Fair Lawn, NJ). Liver triglyceride (TG) and free fatty acid (FFA) levels were determined using commercial kits according to manufacturer's instructions. (Thermo Scientific, Waltham, MA).

2.2.7 RNA Isolation and Real-time RT-PCR

The mRNA levels were assessed by real-time polymerase chain reaction (PCR). In brief, the total RNA was extracted from liver, colon, spleen, epididymal white adipose tissue (eWAT) and lung tissue with Trizol according to manufacturer's protocol (Life Technologies, Carlsbad, CA) and reverse-transcribed using cDNA supermix (QuantaBio, Beverly, MA). Quantitative real-time PCR was performed on an ABI 7500 real-time PCR

thermocycler, whereas SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis. The relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against the housekeeping genes, 18S (mouse) or ACTB (gene name for human Beta-Actin). Relative mRNA expression was calculated using the Ct method. The primer pairs used are listed in Table 2.1.

Table 2.1 List of Primers

Gene name	Forward sequence 5'→3'	Reverse sequence 5'→3'
<i>hmCAM P</i> (LL-37)	ATCATTGCCAG- GTCCTCAG	GTCCCCATACAC- CGCTTCAC
<i>hmACT B</i> (Beta-Actin)	CAAAGACCTGTACGCCAACAC	CATACTCCTGCTTGCTGATCC
<i>hmVDR</i>	GCTTGTCAAAAGGCGGCAG	CCCAAAGGCTTCTGGTCCG
<i>hmHIF 1A</i>	ACTCATCCATGTGACCACG	TAGTTCTCCCCCGGCTAG
<i>Camp</i>	TCTCTACCGTCTCCTGGACCTG	CCACATACAGTCTCCTTCACTCG
<i>18S</i>	GTAACCCGTTGAACCCATT	CCATCCAATCGGTAGTAGCG
<i>Il-1β</i>	TTCATCTTTGAAGAAGAGCCCAT	TCGAGCCTGTAGTGACGTT
<i>Il-6</i>	TGGAAATGAGAAAAGAGTTGTGC	CCAGTTTGGTAGCATCCATCA
<i>Tnf-α</i>	AGGCTGCCCCGACTACGT	GACTTTCTCCTGGTATGAGATAGCAAA
<i>Il-18</i>	AAAGTGCCAGTGAACCC	TTTGATGTAAGTTAGTGAGAGTGA
<i>Mcp-1</i>	ACCACAGTCCATGCCATCAC	TTGAGGTGGTTGTGGAAAAG
<i>Il-10</i>	CCCTTTGCTATGGTGTCTT	TGGTTTCTCTTCCCAAGACC
<i>Lbp</i>	CAAACCTCTGCCAGTCACA	GGACATTGGCACCCAAGT
<i>Cd14</i>	TGAGTATTGCCAAGCACACT	GTAAGTGAGATCCAGCACGCT
<i>Tlr-4</i>	GGAGGGAAGAGGCAGGTGTC	GGGAGGCATCATCTGGCAT
<i>Caspase-1</i>	GAAGAGATGTTACAGAAGCC	CATGCCTGAATAATGATCAC
<i>Nlrp3</i>	TGCCTGTTCTTCCAGACTGGTGA	CACAGCACCTCATGCCCCGG
<i>Hmox-1</i> (HO-1)	CTGTGTAACCTCTGCTGTTCC	CCACACTACCTGAGTCTACC
<i>Nrf2</i>	CGAGATATACGCAGGAGAGGTAAGA	GCTCGACAATGTTCTCCAGCTT

<i>Gsr</i>	GCTATGCAACATTCGCAGATG	AGCGGTAAACTTTTTCCCATG
<i>Cat</i>	TGAGAAGCCTAAGAACGCAATTC	CCCTTCGCAGCCATGTG
<i>Gstp1</i>	TGGGCATCTGAAGCCTTTG	GATCTGGTCACCCACGATGAA
<i>Gpx1</i>	GAAGAACTTGGGCCATTTGG	TCTCGCCTGGCTCCTGTTT
<i>Uox</i>	GAAGTGGAATTTGTCCGAACTGG	CGAAGTTGCCACCTCTTTGAT
<i>Xdh</i>	ATGACCGCCTTCAGAACAAAGA	TGTCACAGCAACATGATGCAA
TM7	CATAAAGGAATTGACGGGGAC	GACCTGACATCATCCCCTCCTTCC
Universal bacteria	AAACTCAAAGKAATTGACGG	TCACRRCACGAGCTGAC
<i>Akkermansia</i>	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGAT
<i>Vdr</i>	GAGGTGTCTGAAGCCTGGAG	ACCTGCTTTCTGGGTAGGT
<i>Hif-1α</i>	GAAATGGCCCAGTGAGAAAA	CTCCACGTTGCTGACTTGA
<i>P2x7</i>	TGGAACCCAAGCCGACGTTGA	CTCGGGCTGTCCCCGGACTT
<i>Fpr2</i>	CACAGGAACCGAAGAGTGTAAG	CACCATTGAGAGGATCCACAG
<i>Cxcr-2</i>	CACCGATGTATACCTGCTGA	ACGCAGTACGACCCTCAAAC
<i>Vcam-1</i>	AATGGGAATCTACAGCACCTTT	ATATCCGTATCCTCCAAAAACT
<i>Ly6G</i>	TGCGTTGCTCTGGAGATAGA	CAGAGTAGTGGGGCAGATGG
<i>Cxcl-2</i>	ATCCAGAGCTTGAGTGTGACG	GTTAGCCTTGCCTTTGTTCAG

2.2.8 Immunoblots

Liver and spleen tissues or cultured cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Waltham, MA). Lysed samples were then centrifuged for 15 min at 12000 x g at 4°C, and the supernatants were collected. The protein concentration was measured by using a bicinchoninic acid assay (BCA) assay kit (Pierce, Thermo Scientific, Waltham, MA). Sample aliquots were boiled for 5 min and equal protein amounts (usually 35-50 µg) were separated by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane. Blots were blocked for 1 h in Tris-buffered saline/Tween 20 (TBST buffer) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4 °C with the different primary antibodies diluted (typically 1:1000) in TBST buffer containing 3%

BSA. Blots were then incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (typically 1:5000 dilution) for 1 h at room temperature. Following treatment with supersignal west HRP substrate (MilliporeSigma, Billerica, MA), protein bands were detected by a ChemiDoc Molecular Imager (Bio-Rad, Hercules, CA).

Quantification for the bands was performed using ImageJ software (National Institutes of Health) and normalized to β -actin or GAPDH. The following primary antibodies were used: CRAMP (Abcam, Cambridge, UK), NLRP3 (Cell signaling, Danvers, MA), Caspase-1 pro and cleaved (Abcam, Cambridge, UK), ASC (Cell signaling, Danvers, MA), pro IL-1 β (Cell signaling, Danvers, MA), Cleaved IL-1 β (Cell signaling, Danvers, MA), β -actin (Cell signaling, Danvers, MA), GAPDH (Cell signaling, Danvers, MA).

2.2.9 ELISA

Protein levels of human LL-37 in patient serum were determined using human LL-37 enzyme-linked immunosorbent assay (ELISA) kit (Hycult Biotech, Wayne, PA). Mouse CRAMP protein levels in serum were determined using mouse Cathelicidin ELISA kit (MyBiosource, San Diego, CA). IL-1 β levels in mice serum and cell culture medium were determined using IL-1 β ELISA kit (Thermo Scientific, Waltham, MA) according to the manufacturers' instructions, respectively.

2.2.10 Endotoxin Assay

Chromogenic Limulus Amebocyte Lysate (LAL) endotoxin kit was used to determine serum LPS levels according to the manufacturer's protocol (Lonza, Basel,

Switzerland). All material used for harvesting blood and measuring endotoxin was pyrogen free.

2.2.11 LPS-binding Assay

LPS-binding assay was performed as previously described ⁸⁹. Briefly, RAW264.7 cells (1×10^6 cells/ml) were incubated with FITC-conjugated LPS ($1 \mu\text{g/ml}$) in the absence or presence of CRAMP peptide (0.1, 0.5, 1, 2, 5, 7.5, $10 \mu\text{g/ml}$) in DMEM containing 10% FBS for 15mins at 37°C . Cells were then washed with PBS, and the binding of FITC-LPS was analyzed by flow cytometry (FACSCanto, BD, Franklin Lakes, NJ). The fluorescence intensity was determined and plotted as fold changes to FITC-LPS control.

2.2.12 Serum ATP

Serum adenosine triphosphate (ATP) level was assessed by a colorimetric/fluorometric assay kit according to the manufacturer's protocol (Biovision, Milpitas, CA). Briefly, the phosphorylation of glycerol generates a colored product, which can be easily quantified by colorimetric methods by reading endpoint absorbance value at $\lambda = 570 \text{ nm}$.

2.2.13 Serum Uric Acid

The levels of uric acid (UA) in mouse serum samples were determined using a commercial UA assay kit (Abcam, Cambridge, UK) according to the manufacturer's protocol. UA level was measured using a colorimetric (at $\lambda = 570 \text{ nm}$) method.

2.2.14 Serum XO Activity

Serum and hepatic xanthine oxidase (XO) activity were determined using a commercial colorimetric kit (Biovision, Milpitas, CA) according to the manufacturer's protocol. Diluted serum samples and liver lysates (collected supernatant only) were used for the assay. In the assay, XO oxidizes xanthine to hydrogen peroxide (H_2O_2) which reacts stoichiometrically with OxiRed probe to generate color (at $\lambda = 570$ nm). Since the color or fluorescence intensity is proportional to XO content, the XO activity can be accurately measured.

2.2.15 Gut Microbiota Analysis

Fecal samples were collected at the harvest from mouse colon and stored at -80°C . Bacterial genomic DNA was then extracted from fecal samples using the MO BIO Powersoil DNA Extraction Kit (Carlsbad, CA). Genomic DNA samples were then analyzed for 16S rRNA Gene Sequencing on the 454 FLX-titanium Platform as previously described ⁷⁹.

2.2.16 Bacterial Cultures

E. coli (*Escherichia coli*), *Bacillus subtilis* 22 CP-1 were cultured in lysogeny broth (LB) (Sigma-Aldrich, St. Louis, MO), while probiotic *Lactobacillus rhamnosus* GG (LGG) was cultured in MRS broth (Sigma-Aldrich, St. Louis, MO), and *Listeria* was cultured in Brain Heart Infusion (BHI) medium (ThermoFisher, Waltham, MA). All bacteria strains were cultured with or without the addition of CRAMP peptide at the concentration of $3\ \mu\text{g/ml}$. Bacteria cultured in 15ml sterilized tubes were incubated at 37°C on an incubation-shaker. Bacterial growth was monitored by measuring optical

density $\lambda = 600$ nm (OD600). OD600 values of bacterial culture were recorded at different time points with 30-minute intervals for a total of 6 hours after CRAMP peptide was added to the bacteria culture. Growth curves of each bacteria strain are depicted accordingly.

2.2.17 Statistics

Statistical analyses were performed using the statistical computer package, GraphPad Prism version 6, (GraphPad Software Inc., San Diego, CA, USA), MS Excel 2016 (Microsoft Corp., Redmond, WA) or SPSS 26.0 (IBM, Chicago, IL). Results are expressed as means $\pm$ standard error of the mean (SEM). Statistical comparisons were made using two-way analysis of variance (ANOVA) with Tukey's *post hoc* test or Student's t-test, where appropriate. Differences were considered to be significant at $p < 0.05$. Columns with different letters are statistically different from each other, or significance may be noted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between groups.

2.3 Results

2.3.1 Dysregulated Cathelicidin in Alcohol Fed Mice and in Patients with ALD

Previous studies in our groups showed that ileal CRAMP mRNA expression was decreased in mice treated with binge alcohol or on chronic alcohol feeding for 4 weeks^{79,80}. To further evaluate CRAMP tissue expression in response to alcohol feeding, the mRNA levels of *Camp* (gene name for mouse CRAMP) in various tissues of the mice fed alcohol were analyzed. CRAMP is highly expressed in the spleen and epididymal white adipose tissue (eWAT) on basal level (Figure 2.1A). Mice received the 24 days chronic

alcohol feeding plus 1 binge at the end of feeding (24D+1B, binge-on-chronic alcohol-feeding paradigm). Alcohol feeding significantly increased *Camp* mRNA expression in the liver and spleen, and decreased expression in the lung tissue, while no change was found in the eWAT and in the colon (Figure 2.1B). Interestingly, spleen CRAMP protein was decreased by alcohol, while liver levels of CRAMP were increased (Figure 2.1C). There was no change in serum CRAMP levels in mice fed alcohol (Figure 2.1D). Similarly, in patients with ALD, the hepatic mRNA levels of LL-37 (gene name *CAMP*) were elevated compared to healthy controls (Figure 2.1E). However, serum LL-37 protein levels were decreased in ALD patients (Figure 2.1E). LL-37 is transcriptionally regulated by hypoxia-inducible factor 1 α (HIF-1 α)^{79,98} and vitamin D receptor (VDR)^{99,100}. Analyzing the expression of HIF-1 α and VDR revealed that hepatic *CAMP* mRNA was positively correlated with mRNA levels of *HIF1A* and *VDR* (Figure 2.1F), indicating likely upregulated hepatic *HIF1A* and *VDR* signaling in patients with ALD.

Figure 2.1 Alcohol exposure induced cathelicidin dysregulation in animals and ALD patients.

(A) Differential expression of *Camp* (gene name of mouse CRAMP) in the liver, colon, spleen, eWAT and lung. (B) Alcohol-induced changes in *Camp* expression in different organs. (C) Alcohol-induced CRAMP protein level changes in the liver and spleen tissue by immunoblotting. (D) Serum CRAMP level. Data are expressed as mean $\pm$ SEM, (n $\geq$ 5). (E) Serum LL-37 (upper panel) and hepatic *CAMP* (gene name of human LL-37) (lower panel) level in ALD patients and healthy controls (HC). Data are expressed as mean $\pm$ SEM, (n $\geq$ 5). (F) Hepatic *CAMP* expression correlates with HIF1A and VDR expression in ALD patients (n=14). *p<0.05, **p<0.01, ***p<0.001.

2.3.2 CRAMP Deficiency Exacerbated Alcohol-induced Liver Steatosis and Injury

To determine the role of CRAMP in ALD, *Camp*^{-/-} and WT mice were fed alcohol in a binge-on-chronic model (24D+1B). Alcohol consumption resulted in increased levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in both WT and *Camp*^{-/-} mice, with more pronounced elevations being found in *Camp*^{-/-} mice (Figure 2.2A). Alcohol feeding increased hepatic fat accumulation in both WT and *Camp*^{-/-} mice, but much more severe macrosteatosis was found in *Camp*^{-/-} mice (Figure 2.2B, upper panel), and this was then confirmed by Oil Red O staining of frozen liver sections (Figure 2.2B, middle panel). Importantly, the distribution of lipid droplets in the livers of *Camp*^{-/-} and WT mice was zonally distinct from each other (Figure 2.2C). Enhanced hepatic fat in *Camp*^{-/-} mice was further confirmed by the measurement of hepatic triglyceride and free fatty acid contents (Figure 2.2D). Moreover, alcohol exposure induced more hepatic apoptosis in *Camp*^{-/-} mice, shown as an increased number of terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL)-positive cells (Figure 2.2B, lower

panel). These results suggest that alcohol feeding exacerbates liver steatosis, injury and hepatic cell death when CRAMP is deficient.

Figure 2.2 CRAMP deficiency exacerbates ethanol-induced liver injury, steatosis and hepatic cell death.

(A) Serum transaminases AST and ALT. (B) Representative photomicrographs of liver sections with H&E (hematoxylin and eosin) staining (200x) (upper panel), Oil-red O staining (200x) (middle panel) and TUNEL-positive cells staining (100x) (lower panel). Black arrows: apoptotic bodies. (C) Zonal distribution of alcohol-induced hepatic fat accumulation. CV: central vein; PT: portal tract; From CV to PT: Liver zones 3, 2, 1. (D) Hepatic triglycerides and free fatty acids in WT and *Camp*^{-/-} mice. Data are expressed as mean ± SEM, (n≥8). *p<0.05, **p<0.01, ***p<0.001.

2.3.3 *Camp*^{-/-} Mice Had Dysbiosis in Response to Alcohol Feeding

Gut bacterial dysbiosis has been well documented in mice with experimental ALD^{61,101-103} and in patients with ALD^{56,60,104,105}. 16S rRNA sequence analysis showed distinctly different bacterial populations in the feces of WT and *Camp*^{-/-} mice fed alcohol. At the phylum level (Figure 2.3A), Bacteroidetes decreased, while Firmicutes increased in *Camp*^{-/-} mice fed alcohol, which agrees with previous studies^{53,106,107}. It is notable that there was a great expansion of Candidatus bacteria (TM7) in alcohol-fed *Camp*^{-/-} mice, which was then confirmed by qRT PCR (Figure 2.3B). TM7 is one of the colitogenic microbiota phyla that have been shown to correlate with non-alcoholic fatty liver disease (NAFLD)^{108,109}. Total bacteria amount in fecal samples of WT and *Camp*^{-/-} mice were comparable (Figure 2.3C). Of note, *Akkermansia*, a genus in the phylum Verrucomicrobia that is important in ALD^{78,79}, was increased in WT mice fed alcohol, while markedly decreased in the *Camp*^{-/-} mice (Figure 2.3D). Genera analysis (Figure 2.3E) showed that *Bacteroides*, which belongs to the Bacteroidetes phylum, completely

disappeared in *Camp*^{-/-} mice fed alcohol. *Thermophagus*, another genus of Bacteroidetes, increased significantly in *Camp*^{-/-} mice by 8-fold. *Oscillibacter* and *Papillibacter* in Ruminococcaceae families were decreased in *Camp*^{-/-} mice, and have been reported to be decreased in cirrhotic patients¹¹⁰. These results suggest that CRAMP may regulate microbiota homeostasis in ALD. Results from *in vitro* study further showed that CRAMP peptide, selectively inhibits bacterial growth. As depicted in Figure 2.3F, CRAMP peptide inhibited the growth of the pathogenic bacteria, *Listeria* and *Escherichia coli* (*E. coli*); however, notably, there was no effect on *Bacillus subtilis* 22 CP-1 and probiotic *Lactobacillus rhamnosus* GG (LGG).

F

Figure 2.3 *Camp*^{-/-} mice had dysbiosis in response to alcohol feeding.

(A) CRAMP deficiency induced microbiota compositional changes on the phylum level as shown by metagenomic analysis using 16S rRNA sequencing in mouse fecal samples ($n \geq 6$). (B) Fold change of Saccharibacteria (TM7) bacteria abundance by alcohol feeding and CRAMP deficiency. (C) Total bacteria fold change by analyzing bacteria 16S rRNA using qRT PCR. (D) *Akkermansia* bacteria fold change. (E) CRAMP deficiency-induced bacteria abundance changes on the genera level. (F) Growth curve of *Listeria*, *E. coli*, *Bacillus subtilis* 22 CP-1 and probiotic LGG. Data are expressed as mean $\pm$ SEM ($n \geq 4$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.3.4 *Camp*^{-/-} Mice Had an Increased Hepatic Pro-inflammatory Response

Inflammation is a hallmark of ALD in animal models and human subjects^{28,111}. *Camp*^{-/-} mice had significantly increased mRNA levels of pro-inflammatory cytokines *Il-1 β* , *Il-6*,

Tnfα and *Il-18*, and the chemokine, *Mcp-1* (Figure 2.4A). Interestingly, *Il-10*, an anti-inflammatory cytokine¹¹², was substantially lowered in the *Camp*^{-/-} mice compared to the WT mice fed alcohol (Figure 2.4A). Histological analysis revealed increased levels of neutrophil infiltration and macrophage activation in the livers of *Camp*^{-/-} mice (Figure 2.4B). These results suggest that lacking CRAMP increased the pro-inflammatory response to alcohol in the liver and was associated with exacerbated neutrophil infiltration and increased macrophage activation.

Figure 2.4 Increased pro-inflammatory response in the liver of *Camp*^{-/-} mouse.

(A) Hepatic cytokine and chemokine mRNA expression. (B) Representative photomicrographs of paraffin-embedded livers stained for CAE (200x) (upper panel) and

frozen liver tissue stained with immunofluorescence (IF) F4/80 antibody (100x) (lower panel). Black arrows: Infiltrated neutrophils; white arrows: F4/80 positive stained macrophages. Blue: DAPI counter stained nuclei; Red: F4/80. (C) Quantification of CAE (upper panel) and F4/80 IF (lower panel) staining. Data are expressed as mean $\pm$ SEM, (n $\geq$ 8). *p<0.05, **p<0.01, ***p<0.001.

2.3.5 CRAMP Deficiency Exacerbated Alcohol-induced IL-1 β Production, Partially Through Enhancement of LPS Signaling

Serum level of IL-1 β was significantly increased in AF *Camp*^{-/-} mice compared to the WT controls, indicating an inflammasome activation (Figure 2.5A), which requires 2 signals for its full activation¹¹³. Serum endotoxin levels in both WT and *Camp*^{-/-} mice were comparable (Figure 2.5B). To examine whether CRAMP is involved in LPS delivery and signal activation, the expression of LPS binding protein (LBP), CD14 and TLR4 was analyzed. *Camp*^{-/-} mice had a significantly increased hepatic mRNA expression of LBP and CD14, and a slight trend of increase in TLR4 (Figure 2.5C), indicating an enhanced LPS activation. To further examine the role of CRAMP on LPS binding in macrophages, RAW264.7 cells were treated with synthetic CRAMP peptide in the presence of fluorescein isothiocyanate (FITC)-labeled LPS. CRAMP peptide inhibited LPS binding to macrophages in a dose-dependent manner (Figure 2.5D). The expression of genes involved in NLRP3 inflammasome activation was then analyzed. Hepatic *Nlrp3* and *Caspase-1* mRNA expression was increased in alcohol-fed *Camp*^{-/-} mice compared to its WT controls. The active form of Caspase-1 protein, Caspase-1 p12, was significantly increased in *Camp*^{-/-} mice (Figure 2.5E). Similar to the increased serum level of IL-1 β protein, cleaved IL-1 β was also elevated in the livers of *Camp*^{-/-} mice

(Figure 2.5F). These findings suggest that the exacerbation of alcohol-induced IL-1 β production in *Camp*^{-/-} mice is likely through the loss of the inhibitory effect of CRAMP on LPS binding to macrophages and through the enhanced inflammasome activation in the liver.

Figure 2.5 CRAMP deficiency exacerbated alcohol-induced IL-1 β production partially through the enhancement of LPS signaling.

(A) Serum concentration of IL-1 β analyzed by ELISA. (B) Serum endotoxin level. (C) Hepatic *Lbp*, *Cd14* and *Tlr-4* on mRNA level. (D) LPS binding activity shown as FITC intensity of FITC-conjugated LPS treated Raw 264.7 cells analyzed by flow cytometry. FITC-LPS: 1 μ g/ml. Significant digits represent the difference comparing FITC-LPS alone treated group. (E) Hepatic mRNA level of *Nlrp3* and *Caspase-1*. (F) Representative bands of immunoblotting (left panel) and quantification (right panel) of molecules in inflammasome activation in whole liver lysates. Data are expressed as mean $\pm$ SEM, (n $\geq$ 8). *p<0.05, **p<0.01, ***p<0.001.

2.3.6 NLRP3 Inflammasome Activation and LPS-induced Inflammatory Cytokine

Expression in Macrophages was Attenuated by Synthetic CRAMP Peptide

Bone marrow-derived macrophages (BMDMs) from both *Camp*^{-/-} and WT mice were treated with synthetic CRAMP peptide in the presence of LPS. LPS treatment significantly increased mRNA expression of *Il-1 β* and *Il-6* in BMDMs from *Camp*^{-/-} mice compared to WT BMDMs (Figure 2.6A). *Il-10* mRNA expression was also marginally increased by LPS in *Camp*^{-/-} BMDMs (Figure 2.6A). CRAMP peptide treatment inhibited LPS-induced expression of inflammatory cytokines, including *Il-1 β* , *Il-18*, *Il-6* and *Tnf- α* . *Il-10* mRNA expression was also inhibited by CRAMP peptide in *Camp*^{-/-} BMDMs. The inhibitory effects of CRAMP peptide at the concentrations of 1 μ g/ml and 10 μ g/ml on LPS-induced cytokine expression are comparable, indicating a sensitization of *Camp*^{-/-} BMDMs to CRAMP treatment in response to LPS stimulation (Figure 2.6A).

Additionally, CRAMP peptide inhibited LPS-induced IL-1 β in the cultural media in both WT and *Camp*^{-/-} BMDMs (Figure 2.6B). To further confirm the observations in BMDMs,

these *ex vivo* experiments were extended to *in vitro* ones using RAW264.7 macrophages. CRAMP peptide significantly reduced the protein level of NLRP3 in a dose-dependent manner (Figure 2.6C). Pro-caspase-1 protein level was moderately reduced by CRAMP peptide. Remarkably, CRAMP peptide completely inhibited the pro-IL-1 β protein expression by LPS stimulation at both high and low doses (Figure 2.6C). Similar to the observations in LPS-treated *Camp*^{-/-} BMDMs, CRAMP peptide treatment down-regulated LPS-induced *Il-1 β* , *Il-6* and *Tnf- α* mRNA expression in a dose-dependent manner (Figure 2.6D). Importantly, the protein level of IL-1 β in the LPS-treated RAW264.7 cell culture medium was decreased by CRAMP peptide treatment (Figure 2.6E).

Figure 2.6 NLRP3 inflammasome activation and LPS-induced inflammatory cytokine expression in macrophages were attenuated by synthetic CRAMP peptide.

(A) LPS-induced inflammatory cytokine expression in WT and *Camp*^{-/-} BMDMs treated with CRAMP peptide. (B) IL-1 β protein concentrations in the culture medium of BMDMs. (C) Immunoblotting of NLRP3 inflammasome complex proteins and quantification in mouse macrophage RAW264.7 cells. (D) LPS-induced inflammatory cytokine mRNA expression in RAW264.7 cells treated by CRAMP peptide. (E) IL-1 β

protein levels in the cultural media of RAW264.7 cells. Data are expressed as mean $\pm$ SEM (n=3). Columns with different letters are significantly different.

2.3.7 CRAMP Deficiency Exacerbated Alcohol-induced Endogenous Danger Signals

Whether CRAMP is involved in the Signal 2 regulation in NLRP3-inflammasome is not clear. ATP and uric acid (UA) have been identified as endogenous danger signals for NLRP3 inflammasome activation in ALD^{114,115}. The serum concentration of ATP and UA in *Camp*^{-/-} mice and their WT controls were measured. Results showed that mice lacking CRAMP had higher serum concentrations of UA and ATP (Figure 2.7A) compared to their WT controls. Importantly, analysis of the banked serum samples from alcohol use disorder (AUD) patients revealed a significant positive correlation between serum IL-1 β and UA concentrations in AUD patients with ALD, but only an insignificant inverse association was observed in AUD patients without ALD (Figure 2.7B). Next, we examined whether treatment with CRAMP peptide can suppress UA-exacerbated IL-1 β production in LPS-primed RAW264.7 macrophages. To exclude the effects of CRAMP peptide on LPS binding, cells were washed with PBS after LPS treatment and immediately exposed to UA and CRAMP in FBS-free media. UA alone had no effect on cellular pro-IL-1 β and cleaved IL-1 β protein levels, which were, however, increased by LPS treatment (Figure 2.7C). Addition of UA to LPS-primed cells showed no effect on cellular pro-IL1 β and cleaved IL-1 β (Figure 2.7C), but significantly increased media IL-1 β protein concentration (Figure 2.7D). Importantly, CRAMP peptide treatment completely inhibited cellular pro-IL-1 β , cleaved IL-1 β expression and the mature IL-1 β concentration in culture media (Figure 2.7C&D). In contrast to RAW264.7 cells, the

addition of UA to LPS-primed BMDMs dramatically increased cellular cleaved IL-1 β expression in both WT and *Camp*^{-/-} mice, which were significantly reduced by CRAMP peptide. Of note, LPS-primed *Camp*^{-/-} BMDMs showed increased levels of Caspase-1 p12 and pro IL-1 β protein level compared to control BMDMs, along with slightly increased levels of cellular cleaved IL-1 β (Figure 2.7E). A similar inhibitory effect of CRAMP peptide on mature IL-1 β production in RAW264.7 culture media was observed in BMDMs; however, no differences were found between WT and *Camp*^{-/-} mice (Figure 2.7F). These results suggested that CRAMP deficiency causes UA dysregulation in ALD subjects that leads to elevated IL-1 β production through exacerbated inflammasome activation.

Figure 2.7 Deficiency of CRAMP exacerbated the alcohol-induced endogenous danger signal, uric acid (UA), which correlates with the upregulated IL-1 β production.

(A) Serum UA and ATP level. Data are expressed as mean $\pm$ SEM, (n $\geq$ 8). *p<0.05, **p<0.01. (B) UA was inversely and insignificantly associated with IL-1 β in alcohol use disorder (AUD) patients without ALD (left panel); UA was positively and significantly associated with IL-1 β in AUD patients with mild ALD (right panel). Data are expressed as mean $\pm$ SEM, (n $\geq$ 15). (C) Immunoblotting (left panel) and quantification (right panel) for Caspase-1 and IL-1 β protein in LPS and UA treated RAW264.7 cells. LPS: 500 ng/ml; UA (Uric acid): 500uM. CRAMP peptide: 10 μ g/ml. (D) IL-1 β protein level in RAW264.7 culture medium. (E) Immunoblotting of BMDM lysates from WT and *Camp*^{-/-} mice. (F) IL-1 β protein level in BMDM culture medium. Data are expressed as mean $\pm$ SEM, (n $\geq$ 4). Columns with different letters are significantly different.

2.3.8 CRAMP Deficiency Exacerbated Alcohol-induced Oxidative Stress

Alcohol metabolism-generated reactive oxygen species is a hallmark in ALD development and progression ¹¹⁶. UA metabolism is regulated by xanthine oxidase (XO), an important enzyme essential for the endogenous production of UA as a final product of purine metabolism. XO activation is upregulated by oxidants, which, in turn, produces more reactive oxygen species (ROS) ¹¹⁷. XO activity was increased significantly in the livers of *Camp*^{-/-} mice fed alcohol ((Figure 2.8A). Immunoblots showed increased CYP2E1 in the livers of alcohol-fed *Camp*^{-/-} mice compared to WT mice, although it was not statistically significant (Figure 2.8B). *Camp*^{-/-} mice had increased DHE staining under control conditions, and alcohol feeding significantly increased hepatic superoxide and hydrogen oxide species, which were markedly elevated in *Camp*^{-/-} mice fed alcohol (Figure 2.8C). These observations were then confirmed in an *in vitro* study using the

mouse hepatocyte cell line, Hepa1-6. Knockdown of CRAMP in Hepa1-6 cells was achieved by transfecting cells with CRAMP specific siRNA (siRNA-*Camp*), while the control cells were transfected with non-targeting siRNA (siRNA-NT). Desferrioxamine (DFO), a Hif-1 α activator, was used as an inducer for *Camp* expression in Hepa1-6 cells. DFO is an iron-chelator that induces hypoxic conditions and, thus, increases Hif-1 α activation, which is positively correlated with *CAMP* expression in ALD patients (Figure 2.1F). As expected, DFO induced a significant increase of *Camp* expression in Hepa1-6 cells transfected with siRNA-NT (Hepa-siR-NT) but not with siRNA-*Camp* (Hepa-siR-*Camp*), confirming that CRAMP was efficiently knocked down (Figure 2.8D). Additionally, Hepa-siR-*Camp* cells had significantly higher mRNA levels of heme oxygenase (HO)-1 (gene name *Hmox-1*) than Hepa-siR-NT cells with or without DFO treatments (Figure 2.8D). These results indicated enhanced oxidative stress when CRAMP was lacking. Next, the expression of genes involved in oxidative stress was analyzed in transfected cells treated with ethanol. Ethanol significantly increased *Hmox-1*, *Nrf2*, *Gsr*, *Cat*, *Gstp1* and *Gpx1* mRNA expression in Hepa-siR-*Camp* cells, while no differences were observed in Hepa-siR-NT cells by ethanol (Figure 2.8E). Importantly, UA concentration in culture supernatant was increased by alcohol exposure in cells lacking CRAMP, but there was no change in cells transfected with non-targeting siRNA (Figure 2.8F). Together, these results suggest that CRAMP deficiency causes exacerbated oxidative stress that partially contributes to the increased level of the danger signal, UA, and inflammasome activation.

Figure 2.8 Deficiency of CRAMP exacerbated alcohol-induced oxidative stress.

(A) Xanthine oxidase (XO) activity was measured in the serum and liver lysates from alcohol-fed mice. (B) Immunoblotting (left panel) and quantification (right panel) for CYP2E1 protein in liver lysates. (C) Liver ROS production (left panel) and quantification (right panel) by DHE staining. Magnification: 100X. Data are expressed as mean $\pm$ SEM, (n $\geq$ 8). *p<0.05, **p<0.01. (D) Validation of knockout efficiency by siRNA-Camp transfection in desferrioxamine (DFO) treated the mouse hepatocyte cell line, Hepa1-6

cells (left panel). DFO-induced oxidative stress in transfected Hepa1-6 cells (right panel). (E) Ethanol induced expression of genes involved in oxidative stress production in Hepa1-6 cells. Ethanol: 400mM, 4hours. (F) Ethanol induced-UA production in Hepa1-6 culture supernatant (fold change). Data are expressed as mean $\pm$ SEM, (n $\geq$ 4). *p<0.05, **p<0.01. Columns with different letters are significantly different.

2.3.9 CRAMP Peptide Administration Reversed Alcohol-induced Liver Steatosis and Injury

Next, we examined whether administration of CRAMP peptide to *Camp*^{-/-} and WT mice could attenuate alcohol-induced liver damage. Three days of synthetic CRAMP peptide administration (Figure 2.9A) marginally suppressed alcohol feeding-increased *Saccharibacteria* abundance and elevated *Akkermansia* abundance in *Camp*^{-/-} mice, while no change was found for the total bacteria load in fecal samples (Figure 2.3D). CRAMP peptide treatment did not alter serum AST levels but did significantly reduce ALT levels in WT mice fed alcohol (Figure 2.9B). In *Camp*^{-/-} mice, both AST and ALT levels were markedly decreased by CRAMP peptide (Figure 2.9B). CRAMP peptide administration robustly reduced alcohol-induced hepatic fat accumulation in both *Camp*^{-/-} and WT mice (Figure 2.9C), and this was confirmed by hepatic triglyceride and free fatty acid contents (Figure 2.9D). CRAMP peptide treatment significantly reduced hepatic mRNA expression of *Il-1 β* and *Mcp-1* in WT and *Camp*^{-/-} mice (Figure 2.9E). In contrast, anti-inflammatory cytokine *Il-10* expression was upregulated by CRAMP peptide in both *Camp*^{-/-} and WT mice (Figure 2.9F).

Figure 2.9 CRAMP peptide administration rescued alcohol-induced liver steatosis and injury.

(A) Illustration of feeding and treatment timeline. (B) Serum transaminases AST and ALT. (C) Representative photomicrographs of liver sections of H&E (100x) and Oil Red O staining (200x). (D) Hepatic level of triglycerides and free fatty acids. (E) Hepatic mRNA expression of *Il-1 β* , *Mcp-1*. (F) Hepatic mRNA expression of *Il-10*. Data are expressed as mean $\pm$ SEM, (n $\geq$ 4). *p<0.05, **p<0.01, ***p<0.001.

2.3.10 CRAMP Peptide Administration Attenuated Alcohol-induced IL-1 β Production via Inhibiting Inflammasome Activation

Serum IL-1 β concentration was marginally decreased in the WT mice but robustly decreased in *Camp*^{-/-} mice by CRAMP peptide (Figure 2.10A), indicating a reduced inflammasome activation. Serum LPS levels were not significantly altered by CRAMP peptide (Figure 2.10B), but *Lbp* and *Cd14* mRNA expression was significantly reduced in both *Camp*^{-/-} and WT mice by CRAMP peptide (Figure 2.10C). In addition, CRAMP peptide dramatically decreased serum UA levels and XO activity in *Camp*^{-/-} mice but only marginally in the WT mice (Figure 2.10D&E).

Figure 2.10 CRAMP peptide administration attenuates alcohol-induced IL-1 β production via inhibiting inflammasome activation signaling.

(A) Serum IL-1 β protein levels. (B) Serum LPS levels. (C) Hepatic mRNA expression of *Lbp*, *Cd14* and *Tlr-4*. (D) Serum UA levels. (E) Serum XO activity. (F) Model for the effects of CRAMP on ALD. Data are expressed as mean $\pm$ SEM, (n=4-8).

2.4 Discussion

Hepatic inflammation is a hallmark of ALD development. Alcohol exposure reduces antimicrobial peptide expression and causes intestinal bacterial dysbiosis, immune dysregulation and gut barrier dysfunction, which, in turn, leads to increased endotoxin release and hepatic bacterial translocation that activate TLR-4 on Kupffer cells and causes liver inflammation. In addition to LPS-induced TLR activation, recent studies demonstrate that endogenous danger molecules are critical in hepatic inflammasome activation in ALD development^{114,115}. Studies in this chapter showed that CRAMP plays a role in both LPS-mediated initiation and UA-mediated maturation of inflammasome activation in mice fed alcohol.

Alcohol exposure induces gut dysbiosis, which is largely attributed to the reduced expression of antimicrobial factors. Reg3 lectins represent an important family of gut-derived AMPs, and depleting *Reg3b* and *Reg3g* exacerbated, while increasing *Reg3g* expression attenuated, gut dysbiosis and alcohol-induced liver injury⁷⁶. A recent study further showed that alcohol feeding caused α -defensin dysfunction, which was critically involved in the pathogenesis of AH in mice⁷⁸. CRAMP is the only member in the cathelicidin family of AMP, and it is transcriptionally regulated by vitamin D receptor and hypoxia-inducible factor 1 α (HIF-1 α). Our recent study demonstrated that intestinal HIF1 α plays an important role in maintaining gut microbiota homeostasis and gut barrier

function by activating barrier-protecting factors, such as CRAMP, β -defensins, cluster of differentiation 73 (CD73), and claudin-1. Depleting HIF-1 α in the intestine exacerbated ALD in mice accompanied by a reduction of CRAMP, while activation of HIF-1 α increased CRAMP and attenuated ALD ⁷⁹. It was hypothesized that CRAMP may represent another important AMP in alcohol-induced gut dysbiosis in ALD. Indeed, alcohol exposure induced a significant decrease of Bacteroidetes and an increase of Firmicutes in *Camp*^{-/-} mice compared to WT mice, as has been demonstrated in previous studies ^{53,106,107}. Ferrere et al. showed that the decreased fecal Bacteroidetes/Firmicutes ratio occurred only in alcohol sensitive mice but not in resistant mice ¹⁰⁷. It is thus possible that CRAMP deficiency contributes to the gut dysbiosis and sensitizes the mice to alcohol exposure, which leading to severe ALD. At the genus level, *Akkermansia* was decreased in *Camp*^{-/-} mice. Although the role of *Akkermansia* in ALD remains elusive, it has been shown that *Akkermansia* abundance declined in both ALD patients and in mice fed chronic alcohol, and supplementation with *Akkermansia* showed a protective effect in ALD ¹¹⁸. Our data suggested a critical involvement of CRAMP in maintaining the homeostasis of gut microbiota by selectively inhibiting bacterial growth in ALD.

Unlike Reg3, which is expressed mainly in the intestine, CRAMP is expressed in multiple organs, including intestine, lung, liver, spleen, and adipose tissue. Alcohol treatment increased hepatic but decreased intestinal CRAMP expression in mice. In contrast to intestinal HIF-1 α ⁷⁹, the role of hepatic HIF-1 α in lipid metabolism in ALD is the subject of conflicting reports ¹¹⁹⁻¹²¹. It is known that alcohol exposure induces hepatic hypoxia ¹²² which then increases HIF-1 α expression and upregulates a variety of genes, including CRAMP, that have either deleterious or protective roles. The induction of

hepatic *Camp* in ALD mice is likely a protective mechanism against alcohol-induced injury, since CRAMP deficiency exacerbated ALD. Agreeing with the study in mice, patients with ALD had higher hepatic *CAMP* mRNA expression.

The depletion of CRAMP in mice fed alcohol led to an exacerbated ALD response accompanied by an increased hepatic inflammasome activation, which requires two signals. LPS activation of TLR4 initiates the Signal one in inflammasome activation, and endogenous, sterile, molecules are required to activate Caspase-1 to produce mature IL-1 β , which kills hepatocytes. Interestingly, although *Camp*^{-/-} mice had dysbiosis in response to alcohol exposure, serum LPS levels were not altered, indicating that CRAMP deficiency-exacerbated ALD is likely independent of gut barrier function. Instead, hepatic mRNA expression of LBP and CD14 were significantly increased in *Camp*^{-/-} mice. LBP is an acute phase protein that presents LPS to surface pattern recognition receptors, such as CD14 and TLR4, to elicit immune responses¹²³. Increased LBP and CD14 in *Camp*^{-/-} mice suggest a functional increase of LPS by increasing LPS binding to its activation complex. Indeed, CRAMP peptide inhibited LPS binding to macrophages in a dose-dependent manner. These results demonstrate that increased CRAMP in the liver in response to alcohol might serve as an inhibitory mechanism for LPS signaling.

One of the most important finding of this study was the significantly elevated serum IL-1 β protein and hepatic *Nlrp3* and *Caspase 1* mRNA expression in *Camp*^{-/-} mice. This indicated an activation of the Signal 2 in alcohol-induced inflammasome activation. Previous research demonstrated that UA or ATP, as Signal 2 molecules, play a causal role in the liver inflammation caused by acetaminophen, ischemia-reperfusion injury and alcohol¹¹⁴. Agreeing with these studies, we showed that serum IL-1 β levels are positively

correlated with levels of UA in patients with ALD, and UA combined with LPS induced a robust elevation of mature IL-1 β levels, further demonstrating the requirement of 2 signals in IL-1 β production. Most importantly, mice lacking CRAMP that were fed alcohol had an elevation of serum UA levels, and CRAMP peptide treatment lowered the UA concentration and blocked the IL-1 β elevation. UA metabolism is regulated by multiple mechanisms. Our data suggest that the elevation of UA in *Camp*^{-/-} mice fed alcohol is likely attributed to the increased XO activity resulting, at least in part, from alcohol-induced oxidative stress. Taken together, our data suggest that CRAMP inhibits inflammasome activation by decreasing UA production in mice with experimental ALD. There are intrinsic mechanisms that can control alcohol-induced liver inflammation. Alcohol-increased hepatic CRAMP expression is likely an adaptive response to inhibit both LPS activation and UA-mediated Signal 2 activation in alcohol-induced inflammasome activation.

The presented data demonstrated that exogenous CRAMP administration reduces alcohol-induced hepatic steatosis, which is in line with previous research showing that lentivirus-mediated CRAMP overexpression reduced hepatic fat accumulation in obese subjects⁸⁸. Which was further demonstrated is that CRAMP peptide reduces alcohol-induced LPS and inflammasome activation by decreasing hepatic *Lbp* and *Cd14* expression and UA production. These findings suggest that CRAMP may represent a novel drug target for treating fatty liver and liver diseases related to hepatic inflammation.

In summary, the studies in this chapter identified the protective role of LL-37/CRAMP in alcohol-induced liver inflammation, steatosis and injury by maintaining

gut microbiota homeostasis and by attenuation of inflammasome activation via inhibiting LPS activation and UA production (Figure 2.10F). The knowledge gained from the present study advances the understanding of the role of CRAMP in alcohol-induced liver injury and help in developing potential therapeutic strategies against ALD.

CHAPTER 3 HIGH FAT FEEDING PRIMES THE LIVER TO ALCOHOL-INDUCED INJURY IN MICE: CRITICAL ROLE OF CATHELICIDIN AS A CHEMOATTRACTANT

3.1 Introduction

3.1.1 The Synergistic Effects of Obesity and Alcohol

Being overweight or obese has long been recognized as one of the most common comorbidities of alcohol-associated liver disease (ALD), and it can have significant effects on the initiation and progression of the disease. As early in 1988, researchers had already associated increased body weight (BW) with liver damage in asymptomatic alcoholic patients, suggesting that overweight is a risk factor for ALD ¹²⁴. The interaction of excessive alcohol drinking and obesity has been further confirmed in many more comprehensive clinical studies, whose subjects included obese alcoholics, non-obese alcoholics and obese non-alcoholics as controls. Higher serum ALT levels were often found in obese alcoholics, and this group of alcoholic subjects usually has a higher risk of progressing from fatty liver (simple steatosis) to advanced forms of ALD, such as steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma ³⁶⁻⁴³.

The detrimental co-effects of alcohol exposure and diet-induced obesity and genetic obesity were explored in studies using rodent models which have revealed underlying mechanisms on how alcohol and obesity work synergistically or additively to

promote development/progression of ALD ^{45,125-132}. Rats fed a choline-deficient (CD) diet and gavaged with commercial whiskey three times per week, including a final binge, for three months had increased liver injury, hepatic steatosis, apoptotic cell death and fibrosis ¹³². Tsukamoto et al. demonstrated early in 1986 that the combination of high fat diet and ethanol can induce liver fibrosis in rats ¹²⁷. A recent study by Dr. Bin Gao's group further elucidated the underlying mechanism that the neutrophils and hepatic stellate cells interact to promote fibrosis in experimental steatohepatitis in mice ¹²⁹. Oxidative stress and nitrosative damage were linked to the synergistic effects of obesity and alcohol consumption in rodents. Feeding rats a HFD for one month followed by gavage with ethanol every 12 h for the last three days increased hepatic oxidative stress but did not increase serum ALT levels ¹²⁵.

In genetically obese *fa/fa* Zucker rats, a binge (gavage) of ethanol every 12 h for three days increased liver injury, steatosis and inflammation when compared to their lean littermates ¹³¹. More mechanistic studies were conducted with genetically obese mice (*ob/ob*). Moderate ethanol binges induced significant liver damage (hepatocyte apoptosis) in *ob/ob* mice by increasing TNF- α and decreasing nuclear NF- κ B activity, which unleashed the apoptotic effects of TNF- α ¹³⁰. Exacerbated hepatic steatosis and injury were seen in a chronic ethanol feeding model with *ob/ob* mice, and the mechanisms is thought to be the impairment of hepatic lipid metabolism mediated by the SIRT1-AMPK signaling pathway ¹²⁶.

Xu et al., using an ASH mouse model, described nitrosative stress mediated by M1 macrophage activation, adiponectin resistance, and accentuated ER and mitochondrial stress as underlying potential mechanisms for synergistic steatohepatitis

caused by moderate obesity and alcohol. In this study, moderate obesity was induced by intragastrically overfeeding of high fat diet, followed with continuous intragastric infusion of alcohol ¹²⁸. Another important mechanism by which HFD-plus-binge ethanol synergistically exacerbate liver injury is through the induction of chemokines and subsequent hepatic neutrophil infiltration. In another experimental alcoholic steatohepatitis (ASH) model that mimics acute ASH in obese binge drinkers, Change et al. showed that feeding mice with short- or long-term HFD plus a single ethanol binge synergistically increased the serum and hepatic free fatty acids, chemokine CXCL-1 expression and exaggerated neutrophil infiltration in the liver, eventually producing severe liver injury ⁴⁵.

3.1.2 Neutrophils and the Innate Immune Response in ALD

Inflammation is a hallmark of ALD and NAFLD/NASH, and has been recognized as a host defense reaction caused by infection or tissue damage in different disease models ^{67,69,133-135}. This host defense is aimed at eliminating the inflammatory stimulus and protecting the surrounding tissue from further damage, thus making it necessary for the survival of the host. Neutrophils are the most abundant leukocytes in circulation and play a critical role in innate immune response. Upon tissue injury, neutrophils are largely increased in number and are recruited to the damaged or infected site. Neutrophils are the first cells to be recruited to sites of damage or infection where they promote innate immunity through phagocytosis, release of antimicrobial products and production of signals that recruit or activate other immune cells ¹³⁶. The inflammatory response represents a prominent example of cell dispersal and clustering that must be tightly

controlled; in another word, a deficient or excessive production of neutrophils in response to damage or infection could also be detrimental or beneficial at the same time ¹³⁷. In bacterial infectious respiratory disease models, selective depletion of the neutrophil cell population results in profound defects in the clearance of bacteria from the lungs, and the repletion of neutrophils in neutropenic mice restores the host defense ¹³⁸. On the other hand, excessive neutrophil presence can be injurious, as it may perpetuate inflammation and cause collateral tissue damage through neutrophil-released enzymes or radicals ¹³⁶.

Clinical studies of AH patients have demonstrated that hepatic expression of several major CXC chemokines, such as *Cxcl-1/Gro-α*, *Il-8*, *Cxcl-5*, and *Cxcl-6*, was upregulated and is correlated with neutrophil infiltration and disease severity ¹³⁹. Similar observations were found in obese patients with nonalcoholic steatohepatitis (NASH) that hepatic expression of *Cxcl-1* and *Il-8* was increased, accompanied by the accordingly upregulated chemokine receptors for *Cxcl-1* and *Il-8* ¹⁴⁰. In this study, hepatic expression of 58 of 222 inflammatory and immune response genes was upregulated in NASH patients, particularly the genes encoding chemokines and chemokine receptors involved in leukocyte recruitment.

In a high fat diet plus ethanol (HFD+E) model, binge alcohol gavage may act as a second hit to induce a more profound increase of neutrophil infiltration in a HFD-primed liver, exacerbating liver damage. Neutrophil infiltration has been implicated in promoting liver damage in various types of liver diseases ¹⁴¹ including drug-induced liver injury ¹⁴²⁻¹⁴⁵ and alcoholic liver injury ^{146,147}. The mechanisms of how the trafficking of neutrophils is regulated under physiologic and pathologic conditions have been explored ¹⁴⁸. Neutrophil infiltration is mediated by multiple adhesion molecules, chemokines and

cytokines, or the so-called classic chemoattractant ¹⁴⁹. Several factors are endothelium-expressed molecules, such as E-selectin, P-selectin, vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Neutrophil-expressed proteins also participate in this process, such as L-selectin (CD62L), E-selectin ligand 1 (ESL-1), P-selectin glycoprotein ligand 1 (PSGL-1), CD44, and members of the CXC chemokine family including chemokine (C-X-C motif) ligand-1 (CXCL-1), CXCL-2 and CXCL-8 (IL-8) ^{137,150}. IL-8/CXCL-8 was the first identified chemokine early in 1987 ¹⁵¹. This has led to the cloning of several chemokine receptors, including CXCR-1 and CXCR-2. CXCR-1 is highly selective for IL-8/CXCL-8, whereas CXCR-2 is responsive to IL-8 and other CXC chemokines. CXCR-2 is a G protein coupled chemokine receptor expressed on neutrophils ^{137,152}. In mice, CXCR-2 and another chemokine receptor, CXCR-4, play major roles in regulating neutrophil trafficking. High CXCR-4 expression retains immature neutrophils in the bone marrow while high CXCR-2 expression allows the mobilization of mature neutrophils in the blood and their subsequent recruitment to inflamed sites in response to transient CXCR-4 inhibition ¹⁵³.

CXCL-1 and CXCL-2 are the two most studied ligands of CXCR-2 and are potent neutrophil chemoattractants required for the mature neutrophils trafficking ^{139,152,154-157}. A recent study revealed a critical role of CXCL-1 in HFD and alcohol consumption-induced liver injury and inflammation. Chang et al. analyzed the hepatic expression of an array of adhesion molecules, chemokine and cytokines using a mouse HFD+E model, and found that *Cxcl-1* increased up to 20-fold and 30-fold in the liver after 3 days of HFD and 3 months of HFD+E feeding, respectively ⁴⁵. This is consistent with clinical observations that hepatic *Cxcl-1* markedly increased in AH patients, and is positively correlated with

hepatic neutrophil infiltration ¹³⁹. The authors showed that HFD increases free fatty acids (FFAs) in the liver and serum and up-regulates hepatic CXCL-1 expression, resulting in an exaggerated neutrophil infiltration in the liver and eventually exacerbated liver injury ⁴⁵.

3.1.3 Cathelicidins: Chemoattractants for Neutrophil Infiltration

Other molecules have also been identified to bind CXCR2 in terms of initiating and directing neutrophil infiltration. The human AMP cathelicidin, LL-37, has been described as a chemoattractant ^{92,93} and may act as a functional ligand for CXCR2 on human neutrophils. Zhang et al. demonstrated that LL-37 was shown to function similarly to the CXCR-2-specific chemokine, CXCL-1, in terms of receptor down-regulation and intracellular calcium mobilization on freshly isolated neutrophils. IL-8/CXCL-8 is a chemokine that binds both CXCR-1 and CXCR-2; when neutrophils were pretreated with IL-8/CXCL-8, the calcium mobilization was completely blocked in neutrophils in response to LL-37. Similar inhibitory effects accompanied by the subsequent blockade of neutrophils migration were observed in neutrophils treated with a selective CXCR2 antagonist. Moreover, CXCR2, but not CXCR1, was internalized in LL-37-treated neutrophils, further confirming the specificity of LL-37 as a functional ligand for CXCR2 ¹⁵⁸.

Cathelicidins, defensins and REG3 lectins are 3 major AMP families that have been extensively studied in different disease models, including metabolic diseases such as ALD ^{76,78,159}. The cathelicidin family is composed a number of multifunctional cationic AMPs which are also known as host-defense peptides (CHDP) ¹⁶⁰. LL-37 is the only

human cathelicidin that has been identified, and it has a murine ortholog named CRAMP (Cathelicidin-related antimicrobial peptide). In addition to microbicidal potential¹⁶¹⁻¹⁶³, LL-37/CRAMP also showed capacity to modulate inflammation and immunity^{87,164-167}. Sun et al. demonstrated that pancreas-produced CRAMP plays a crucial role in T cell function in autoimmune diabetes, and the expression of CRAMP is regulated by gut microbiota⁸⁷. In addition to CXCR-2, LL-37/CRAMP has also been reported to exert chemotactic functions through other membrane receptors, such as formyl peptide receptor-like 1/2 in mice (FPR1/FPR2). De et al. reported that LL-37 is chemotactic for human monocytes and FPR1-transfected human embryonic kidney 293 cells. LL-37 can induce Ca²⁺ mobilization in monocytes, and this can be cross-desensitized by an FPR1-specific agonist. Moreover, this group showed that LL-37 utilizes FPR1 as a receptor to chemoattract human neutrophils and T lymphocytes, which are known to express FPR1⁹³. Kurosaka et al. showed later on that the mouse cathelicidin, CRAMP, just as LL-37, is also chemotactic for human monocytes, neutrophils, macrophages and mouse peripheral blood leukocytes through FPR2. Importantly, an injection of CRAMP into mouse ear punches resulted in the recruitment of neutrophils and monocytes, providing *in vivo* evidence that CRAMP acts as a chemoattractant factor. Moreover, the simultaneous administration of CRAMP and ovalbumin as an experimental antigen to mice, promoted both humoral and cellular immune responses in mice, suggesting that CRAMP also acts as an alarmin in response to danger signals and contributes to both innate and adaptive immunity of the host¹⁶⁸.

Another proposed receptor for LL-37/CRAMP is P2X purinoceptor 7 (P2X7). P2X7 receptor is a trimeric ATP-gated cation channel found predominantly on immune

cells. The activation of P2X7 results in the release of proinflammatory mediators and cell proliferation and death. A recent study demonstrated that LL-37 is internalized by human macrophages, and this is a time-, dose-, temperature-, and peptide sequence-dependent endocytotic process through P2X7. In macrophages pretreated with P2X7 antagonists, significantly less internalized LL-37 was detected. The internalized LL-37 was further shown to traffic to endosomes and lysosomes and contribute to intracellular clearance of bacteria by human macrophages, which synchronized with increased reactive oxygen species and lysosome formation. Importantly, the researchers showed that human macrophages have the potential to import LL-37 released from activated human neutrophils¹⁶⁹. The interaction between P2X7 receptors and cathelicidins has been reported to be species-specific, in some cases (human) positive while in others (mouse) negative^{170,171}.

CXCR-2 has been found to be the most specific receptor for LL-37/CRAMP to stimulate the immune responses in neutrophils. Although FPR2 and P2X7 have been recognized as LL-37/CRAMP receptors in neutrophils, macrophage and monocytes, a study analyzed 21 neutrophils receptors showed that only CXCR-2 was down-regulated by LL-37. Furthermore, a CXCR-2 inhibitor, SB225002, inhibited LL-37-stimulated migration of monocytes, while a FPR2 ligand, peptide WKYMVm, failed to do so¹⁵⁸. This highlights the critical role of cathelicidins as chemoattractant in neutrophils infiltration.

The previous studies showed that CRAMP deficiency exacerbated binge-on-chronic alcohol-induced liver injury, and this is through the regulation of gut microbiota composition and the inhibition of inflammasome activation via inhibiting LPS delivery to

the macrophages and inhibiting uric acid production and signaling. In this chapter, it was aimed to investigate the role of CRAMP as chemoattractant in alcohol-induced liver injury in HFD-fed mice, and to explore the underlying mechanism(s).

3.2 Materials and Methods

3.2.1 Animals

CRAMP KO (*Camp*^{-/-}) male mice (Jackson Laboratory, Bar Harbor, ME) aged 8-10 weeks were used with their age-matched littermates WT mice. Mice were maintained at 22 °C with a 12 h light/dark cycle and had free access to a normal chow diet and tap water. WT and *Camp*^{-/-} mice were subjected to adjusted calories control diet (CD) (10% total kcal from fat) or adjusted calories high fat diet (HFD) (42% total kcal from fat) or feeding for 10 weeks, followed by one binge alcohol gavage for the alcohol group (HFD +E) or maltose dextrin gavage in substitution of ethanol for the control group (CD / HFD). Timelines of experiments and mouse chow composition are depicted in Figure 3.1. During the 10-week feeding period, body weight and food consumption in each feeding group were recorded weekly. At the end of the 6th and 10th week, an oral glucose tolerance test (OGTT) was conducted in each CD or HFD feeding group. At the time of harvest, a portion of the liver (left lateral liver lobe) and spleen tissue were collected in ice-cold DMEM cell culture medium which was supplemented with 2% FBS (fetal bovine serum) immediately after opening of the abdominal cavity for flow cytometry analysis. The serum and the tissues of the liver, spleen, intestine, epididymal white adipose tissue (eWAT), and fecal samples and cecum contents were collected.

3.2.2 ALT and AST Measurements

Serum activity levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using standard kits (Thermo Scientific, Waltham, MA) following the manufacturer's instructions.

3.2.3 Histological Analysis

Liver tissue and epididymal white adipose tissue (eWAT) were collected and fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin-embedded liver tissue blocks were then sliced at 5 μ m thickness and eWAT tissue sliced at 6 μ m on a microtome. The sliced tissues were then floated in a water bath containing distilled water at 45°C for the liver tissue and at 40°C for the adipose tissue. Sections were then transferred onto glass slides. Slides were air dried for 2 days or dried in a 37°C incubator overnight before the day of staining. Otherwise, they were stored at room temperature for future use. Slides were deparaffinized with Citrisolv (Decon, King of Prussia, PA) and rehydrated by immersing in graded ethanol solutions before staining.

H&E Staining Hematoxylin and eosin (H&E) were used to stain the nuclear and cytoplasm components, respectively. After staining, sections were then dehydrated through graded alcohol, cleared in Citrisolv and then mounted with Cytoseal Xyl (Thermo Scientific, Waltham, MA) and observed by microscopy.

CAE Staining Infiltration of neutrophils in the liver was assessed by chloroacetate esterase (CAE) staining according to manufacturer's instructions (Naphthol AS-d Chloroacetate Kit, Sigma, St. Louis, MO). Briefly, liver sections were incubated in a solution of naphthol AS-D chloroacetate, the naphthol AS-D chloroacetate is

enzymatically hydrolyzed by a “specific esterase” in neutrophils, liberating a free naphthol compound. This then couples with a diazonium compound, forming highly colored deposits at sites of enzyme activity that can be visualized under microscopy. Numbers and areas of neutrophil clusters were quantified as previously described ¹⁷².

Ly6G & CRAMP co-localization Staining Following deparaffinization, antigen retrieval was performed on slides in 10mM citrate buffer (pH 6.0) for 20 min at 95°C. Blocking buffer containing 10% fetal bovine serum (FBS) in PBS (Sigma, St. Louis, MO) was added onto the slides and the slides were incubated in a humidified chamber at room temperature for 1 hour, for the blocking of non-specific antibody binding. Sections were then incubated with primary antibody Ly6G (rat-anti-mouse), Cathelicidin (mouse CRAMP, rabbit-anti-mouse) (Abcam, Cambridge, UK) at a concentration of 1:200 in 10% FBS and incubated in a humidified chamber overnight at 4°C. On the next day, slides were washed with PBS three times, 1 minute/time before being incubated with AlexaFluor-conjugated secondary antibodies (Life technologies, Carlsbad, CA). AlexaFluor 488 (Green) (donkey-anti-rat) and AlexaFluor 594 (Red) (goat-anti-rabbit) at a concentration of 1:200 in 10% FBS were used to incubate the slides for 1.5 hours. DAPI (Invitrogen, Carlsbad, CA) was used for nuclei counterstaining. The fluorescence was examined under confocal microscopy and the intensity of fluorescence was quantified using Image J.

3.2.4 Lipid Accumulation

Liver tissues were homogenized in 50mM NaCl (Sigma, St. Louis, MO) solution in distilled water. The homogenates were then used for lipid extraction using methanol

(Fisher chemical, Fair Lawn, NJ) and chloroform (Fisher chemical, Fair Lawn, NJ). Liver triglycerides (TG) and free fatty acid (FFA) levels were determined using commercial kits according to the manufacturer's instructions. (Thermo Scientific, Waltham, MA).

3.2.5 RNA Isolation and Real-time RT-PCR

The mRNA levels were assessed by real-time polymerase chain reaction (PCR). In brief, the total RNA was extracted from the liver and spleen tissue with Trizol reagent according to the manufacturer's protocol (Life technologies, Carlsbad, CA) and reverse-transcribed using cDNA supermix (QuantaBio, Beverly, MA). Quantitative real-time PCR was performed on an ABI 7500 real-time PCR thermocycler, whereas SYBR green PCR Master Mix (Applied Biosystems, Foster City, CA) was used for real-time PCR analysis. The relative quantities of target transcripts were calculated from duplicate samples after normalization of the data against the housekeeping gene, *18S* (mouse). Relative mRNA expression was calculated using the Ct method. The primer pairs used were listed in Table Primers list.

3.2.6 Immunoblots

Liver and spleen tissues were lysed in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Waltham, MA). Lysed samples were then centrifuged for 15 min at 12000 x g at 4°C, and the supernatants were collected. The protein concentration was measured by using a bicinchoninic acid assay (BCA) assay kit (Pierce, Thermo Scientific, Waltham, MA). Sample aliquots were boiled for 5 min and equal protein amounts (usually 35-50 µg) were separated by SDS-PAGE. Proteins were then

transferred to a nitrocellulose membrane. Blots were blocked for 1 h in Tris-buffered saline/Tween 20 (TBST buffer) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4 °C with the different primary antibodies diluted (typically 1:1000; β -actin at 1:10000) in TBST buffer containing 3% BSA. Blots were then incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (typically 1:5000 dilution) for 1 h at room temperature. Following treatment with supersignal west HRP substrate (MilliporeSigma, Billerica, MA), protein bands were detected by a ChemiDoc Molecular Imager (Bio-Rad, Hercules, CA). Quantification for the bands was performed using Image J software (National Institutes of Health) and normalized to β -actin. The following primary antibodies were used: CRAMP (Abcam, Cambridge, UK) and β -actin (Cell signaling, Danvers, MA).

3.2.7 Serum CRAMP and CXCL-1 ELISA

Mouse serum CRAMP protein levels were determined using mouse Cathelicidin ELISA kit (MyBiosource, San Diego, CA) and serum CXCL-1 levels in murine serum were determined using CXCL-1 ELISA kit (R&D, Minneapolis, MN) according to the manufacturers' instructions, respectively.

3.2.8 Flow Cytometry

Hepatic leukocytes preparation: Hepatic leukocytes were prepared as previously described¹⁷³. Briefly, a portion of the liver tissue was collected immediately upon harvest and was homogenized with a 70- μ m pore filter in complete DMEM with 2% FBS on ice. The homogenates were then centrifuged at 2300 rpm for 15 mins. After several washes,

the homogenates were resuspended in a 33% Percoll gradient at 22°C and centrifuged at 2300 rpm for 20 min. The supernatant with the upper layer which contains the dead hepatocytes was discarded, and the cell pellet was collected. Red blood cells (RBCs) were then removed using red cell lysis buffer followed by a wash with 1xPBS and centrifugation.

Flow cytometry analysis: Isolated hepatic leukocytes were blocked by incubating with CD32 antibody, then labeled with antibodies using standard procedures, as described previously ¹⁷⁴. Gr-1, CD11b, Ly6C and F4/80 antibodies that are conjugated with PerCP, APC, FITC and PE from eBioscience (San Diego, CA) were used. Cells were analyzed by flow cytometry (FACSCanto, BD, Franklin Lakes, NJ). Analysis was performed using FlowJo Software (Tree Star, Ashland, OR).

3.2.9 Oral Glucose Tolerance Test (OGTT)

At week 6 and week 10, OGTT were performed for both CD and HFD fed WT and *Camp*^{-/-} mice. Methods used are as previously described ¹⁷⁵. Briefly, mice were fasted overnight for about 16 hours and weighed to calculate the glucose dose at 2.5 g/kg body weight. The working glucose solution was prepared at a concentration of 250 mg/ml in advance. Blood glucose levels of each mouse were determined by glucometer in tail vein blood before the glucose gavage (T0) and at 15 (T15), 30 (T30), 60 (T60) and 120 (T120) minutes following. Tail snipping is used to get blood. Before snips, the tail end was dipped into Bupivacaine (0.25%) for local anesthesia to reduce pain.

3.3 Statistics

Statistical analyses were performed using the statistical computer package, GraphPad Prism version 6, (GraphPad Software Inc., San Diego, CA, USA), MS Excel 2016 (Microsoft Corp., Redmond, WA) or SPSS 26.0 (IBM, Chicago, IL). Results are expressed as means $\pm$ standard error of the mean (SEM). Statistical comparisons were made using two-way analysis of variance (ANOVA) with Tukey's *post hoc* test or Student's t-test, where appropriate. Differences were considered to be significant at $p < 0.05$. Columns with different letters are significantly different, or significance may be noted as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between groups.

3.4 Results

3.4.1 Body Weight Change & Adipocyte Enlargement by HFD+E Treatment

Ten-weeks of HFD feeding (Figure 3.1A & B) markedly increased the body weight of both WT and *Camp*^{-/-} mice over time compared to those animals pair fed with a control diet (CD). However, the increase of body weight was more pronounced in WT mice (127.7% by CD vs 183.2 % by HFD) compared to *Camp*^{-/-} mice (111.7 % by CD vs 152.7 % by HFD). Importantly, *Camp*^{-/-} mice grew much slower overall compared to WT mice, in both control diet and high fat diet feeding groups (Figure 3.2A). By monitoring the daily food intake in absolute amount (g) and caloric amount (Kcal), we found that WT mice, but not *Camp*^{-/-} mice, fed HFD consumed much more food compared to those fed a control diet. Overall, *Camp*^{-/-} mice consumed much less food than WT mice (Figure 3.2B). The differences in body fat mass were then evaluated by determining the ratio of epididymal adipose tissue weight (eWAT) to body weight. The combination of HFD

feeding and binge ethanol gavage (HFD+E) significantly increased the absolute weight of eWAT and the ratio of eWAT/body in both WT and *Camp*^{-/-} mice. No difference was found in the eWAT/body ratio between WT and *Camp*^{-/-} mice. However, the absolute weight of eWAT was significantly lower in *Camp*^{-/-} mice fed with either CD+E or HFD+E (Figure 3.2C). Histological analysis of the adipose tissue showed increased adipocyte size in both WT and *Camp*^{-/-} mice by HFD+E feeding, with more pronounced adipocyte enlargement in WT mice (Figure 3.2D). These results indicated that CRAMP deficiency reduced body weight gain, adipose tissue weight and adipocyte enlargement in mice fed with high fat diet and binge ethanol gavage.

A

B

	CD: Envigo TD06416		HFD: Envigo TD88137	
	% By weight	% Kcal from	% By weight	% Kcal from
Protein	18.6	20.0	17.3	15.2
Carbohydrate	64.7	69.8	48.5	42.7
Fat	4.2	10.2	21.2	42.0
Cholesterol	None		0.2%	
Kcal/g	3.7		4.5	

Figure 3.1 Timelines of experiments & mouse chow composition.

(A) Schematic illustration of experimental design: mouse strain, feeding type and duration, measurements during the feeding period. OGTT: oral glucose tolerance test. (B) Food composition of high fat diet (HFD) and control diet (CD) used.

Figure 3.2 Body weight change & adipocyte enlargement by HFD+E treatment.

(A) Body weight change in percentage (left panel) and in absolute amount (right panel) from the start of feeding period. (B) Food consumption per mouse per day as in absolute amount (left panel) and as in Kcal (right panel). (C) eWAT weight to body weight ratio (left panel) and the absolute amount (right panel). (D) H&E staining of paraffin-embedded eWAT tissue (left panel) and quantified adipocyte size (right panel). Magnification: 100X. Data are expressed as mean $\pm$ SEM, (n $\geq$ 5). *p<0.05, **p<0.01, ***p<0.001.

3.4.2 The Effects of CRAMP Deficiency on Fasting Glucose Concentration and Glucose Intolerance

To determine whether CRAMP deficiency affects glucose homeostasis, oral glucose tolerance tests (OGTT) were performed at week 6 and week 10. Fasting glucose concentrations were not significantly different between CD and HFD-fed WT mice at week 6. However, HFD feeding significantly increased fasting serum glucose level in *Camp*^{-/-} mice, compared to their CD feeding controls and HFD feeding WT controls (Figure 3.3A). HFD itself did not increase glucose intolerance at week 6 in either WT or *Camp*^{-/-} mice. Surprisingly, *Camp*^{-/-} mice showed increased glucose intolerance compared to their WT controls, and this was independent of the CD or HFD feeding (Figure 3.3B). The fasting serum glucose level in WT mice fed HFD significantly increased by week 10, while HFD-fed *Camp*^{-/-} mice had comparable fasting glucose levels to that of week 6 (Figure 3.3 B,C). OGTT revealed that glucose tolerance was substantially decreased by HFD feeding in WT but not *Camp*^{-/-} mice at week 10 (Figure 3.3D). These results indicated that HFD feeding increased fasting glucose concentrations in *Camp*^{-/-} mice at

early stage but improved tolerance at a late stage. In contrast, HFD increased fasting glucose level and reduced glucose tolerance in WT mice over time.

Figure 3.3 The effects of CRAMP deficiency on fasting glucose concentration and glucose intolerance.

Fasting serum glucose of CD+E/HFD+E fed WT and *Camp*^{-/-} mice at week 6 (A) and week 10 (C). Oral glucose tolerance test (OGTT) at week 6 (B) and week 10 (D). Blood glucose level before (time 0) and 15, 30, 60, 120 mins after glucose gavage were shown.

The lower panel showing the quantification of area under curve (AUC) of OGTT at week 6 (left panel) and week 10 (right panel). Data are expressed as mean $\pm$ SEM, (n $\geq$ 5).

*p<0.05, **p<0.01, ***p<0.001.

3.4.3 Alcohol Induced Less Liver Injury and Fat Accumulation in HFD-fed *Camp*^{-/-} Mice

Ten-week HFD+E treatment markedly induced liver injury in WT mice, as reflected by significantly increased serum levels of AST and ALT. Ten-week HFD plus acute alcohol did not alter the serum AST levels, but did increase ALT levels in *Camp*^{-/-} mice. Depletion of CRAMP markedly reduced HFD+E induced liver injury as shown by decreased levels of serum ALT and AST (Figure 3.4A & B). Liver/body weight ratio dramatically increased under HFD+E conditions in WT mice, whereas no significant differences between HFD+E and CD+E were observed in *Camp*^{-/-} mice. Lack of CRAMP significantly decreased liver/body ratio in HFD+E fed group (Figure 3.4C). Histological analysis revealed dramatically increased liver macrosteatosis in WT mice by HFD+E, while only mild microsteatosis was found in the livers of *Camp*^{-/-} mice (Figure 3.4D). Confirming these results, significantly increased levels of liver triglyceride, free fatty acid and total cholesterol in WT mice by HFD+E were found, which were attenuated in *Camp*^{-/-} mice (Figure 3.4E & F). Collectively, CRAMP deficiency decreased HFD+E treatment-induced liver injury and hepatic lipid accumulation.

Figure 3.4 *Camp*^{-/-} mice had decreased levels of HFD+E-induced liver injury and steatosis.

Serum level of liver transaminases AST (A) & ALT (B). (C) Liver to body weight ratio. (D) H&E staining of paraffin-embedded liver tissue. Magnification: 100X. (E) Liver triglycerides contents. (F) Hepatic free fatty acids and cholesterol levels. Data are expressed as mean $\pm$ SEM, (n $\geq$ 5). *p<0.05, **p<0.01, ***p<0.001.

3.4.4 High Fat Feeding Increased Hepatic CRAMP Protein Level While Decreased CRAMP Protein Level in the Spleen

Hepatic CRAMP expression induced by acute ethanol in HFD-fed WT mice were examined. Hepatic *Camp* mRNA was not affected by HFD+E (Figure 3.5A), but the protein levels of CRAMP significantly increased in the liver (Figure 3.5B). Two known transcription factors for *Camp* expression, vitamin D receptor (*Vdr*) and hypoxia inducible factor-1 α (*Hif-1 α*) were analyzed. HFD+E increased the expression of *Vdr* mRNA in WT mice 20 folds compared to the CD+E fed group, and this induction was not observed in *Camp*^{-/-} mice. No changes were found in hepatic *Hif-1 α* either by HFD+E feeding or CRAMP depletion (Figure 3.5C). *P2x7* and *Fpr2*, genes encoding two documented receptors for cathelicidin LL-37/CRAMP, were not affected by HFD+E feeding in WT mice (Figure 3.5D). LL-37 has been reported to act as a functional ligand for the chemokine receptor CXCR2 on human neutrophils¹⁵⁸. Acute ethanol significantly up-regulated *Cxcr-2* in HFD-primed liver in WT but not *Camp*^{-/-} mice (Figure 3.5E). Importantly, *Camp*^{-/-} mice fed with HFD+E had significantly reduced *Cxcr-2* mRNA expression. Additionally, HFD+E treatment induced an induction of another chemokine receptor vascular cell adhesion molecule 1 (*Vcam-1*) in WT mice, but not in *Camp*^{-/-} mice (Figure 3.5E), and *Camp*^{-/-} mice had significantly reduced *Vcam-1* mRNA by HFD+E

treatment. Immunofluorescence staining of liver tissue from WT mice fed CD+E or HFD+E revealed a colocalization of CRAMP and Ly6G-positive neutrophils, which were both increased by HFD+E feeding (Figure 3.5F). These results indicated that there is an enhanced neutrophil infiltration and chemokine expression by acute ethanol in HFD-primed liver, which is possibly CRAMP-dependent. Previously it was showed that CRAMP is expressed predominantly in mouse spleen under physiological conditions, thus the expression of CRAMP in the spleen in the HFD+E model was evaluated. Surprisingly, *Camp* mRNA expression was not changed (Figure 3.5G), but CRAMP protein was significantly reduced in the spleens of WT mice following 10 weeks of HFD plus acute alcohol (Figure 3.5H). Additionally, spleen *Vdr* (Figure 3.5I) increased dramatically, while the mRNA expression of chemokine receptor CXCR-2 decreased significantly in WT mice (Figure 3.5J), indicting a possible organ-organ interaction in terms of CRAMP signaling.

Figure 3.5 High fat feeding increased hepatic CRAMP protein level while decreased CRAMP protein level in the spleen.

(A) Hepatic *Camp* expression. (B) Immunoblotting (left panel) and quantification (right panel) for CRAMP protein in mouse liver whole lysates. (C) Hepatic *Vdr* and *Hif-1α* mRNA expression. (D) Hepatic *P2x7* and *Fpr2* mRNA expression. (E) Hepatic *Cxcr-2* and *Vcam-1* mRNA expression. (F) Immunofluorescence (IF) co-staining of Ly6G (Green) and Cathelicidin (Red) antibodies. Blue: DAPI for nucleus staining. (G) Spleen *Camp* expression. (H) Immunoblotting (left panel) and quantification (right panel) for

CRAMP protein in mouse spleen whole lysates. Spleen *Vdr* (I) and *Cxcr-2* (J) mRNA expression. Data are expressed as mean $\pm$ SEM (n $\geq$ 5). *p<0.05

3.4.5 HFD Increased Inflammatory Monocytes and Neutrophils in the Liver of WT Mice

HFD+E treatment induced an upregulation of CRAMP in the livers of WT mice, and this is accompanied by increased mRNA expression of two chemokine receptors. Whether HFD itself could induce infiltration of inflammatory cells in the liver was not explored. Hepatic leukocytes and spleen lymphocytes were isolated and stained as described in materials and methods, fixed and analyzed by flow cytometry. Immature myeloid cells play a crucial role in mediating inflammatory hepatic steatosis in a HFD mouse model and in *ob/ob* mice ¹⁷³. The results showed that HFD feeding induced a 3-fold increase in the percentage of CD11b⁺Gr-1⁺ immature myeloid cells in the liver compared to CD feeding in WT mice. Another 2-fold increased subpopulation in the CD11b⁺ cells with an intermediate intensity stained Gr-1 (CD11b⁺Gr-1^{int}) was also detected in the livers of WT mice fed HFD (Figure 3.6A).

Gr-1 is comprised of two components: Ly6C and Ly6G. Ly6G is exclusively expressed on neutrophils. Ly6C is expressed on a variety of cells but is most commonly used to distinguish monocyte subtypes. The inflammatory monocyte subset is Ly6C(hi), neutrophils and eosinophils are Ly6C(int), and the 'patrolling' monocyte subset is Ly6C(lo). Therefore, CD11b⁺Gr-1⁺ cells include monocytes, neutrophils, and eosinophils, while CD11b⁺Gr-1⁻ cells represent Ly6C(lo) monocytes. The CD11b⁺ population was then further characterized by gating cells with anti-Gr-1 and anti-Ly6C antibodies. Two major subtypes were identified, CD11b⁺Gr-1⁺ Ly6C(int) neutrophils and CD11b⁺Gr-1⁺

Ly6C(hi) inflammatory monocytes. HFD induced a significant increase in the populations of both neutrophils and inflammatory monocytes in the liver and the spleen. (Figure 3.6B). Collectively, HFD induced the accumulation of immature myeloid cells in the livers of WT mice. Upon further characterization of the CD11b⁺Gr-1⁺ subpopulation, it was demonstrated that infiltrated CD11b⁺Gr-1⁺ Ly6C(int) neutrophils and CD11b⁺Gr-1⁺ Ly6C(hi) inflammatory monocytes were increased by HFD in the liver and spleen of WT mice.

Figure 3.6 HFD feeding increased inflammatory monocytes and neutrophils in the livers of WT mice.

(A) Flow cytometry analysis of CD11b and Gr-1 positive leukocytes in the liver. Cell population labeled CD11b⁺Gr-1⁺ indicating immature myeloid; CD11b⁺Gr-1^{int} indicating another subpopulation. (B) Flow cytometry analysis of gated CD11b and Gr-1 positive liver leukocytes by antibodies Ly6C. CD11b⁺Gr-1⁺ Ly6C(int) neutrophils and CD11b⁺Gr-1⁺ Ly6C(hi) inflammatory monocytes were shown.

3.4.6 HFD+E Treatment Enhances Neutrophil Clustering and Chemokine Expression in the Livers of WT Mice but not in *Camp*^{-/-} Mice

Next, we examined if the increased neutrophil infiltration in the livers of mice fed HFD+E is CRAMP-dependent. CAE staining showed a robust reduction of neutrophil infiltration in *Camp*^{-/-} mice fed with HFD+E compared to their WT controls (Figure 3.7A). Interestingly, the infiltrated neutrophils in the livers of CD+E fed WT mice scattered whereas neutrophils formed clusters in the livers of HFD+E fed WT mice (differentiated by black arrows and yellow arrow, respectively). However, this clustering of neutrophils was almost not detected in HFD+E fed *Camp*^{-/-} mice (Figure 3.7A). Additionally, the number of neutrophil clusters and the area of formed clusters were significantly less and smaller in *Camp*^{-/-} mice compared to WT mice (Figure 3.7B). This indicated that CRAMP may play a role in the recruitment and cluster formation of neutrophils under a HFD+E challenge. In addition, hepatic mRNA expression of Ly6G and chemokines, Mcp-1 and Cxcl-2, was significantly increased in the livers of HFD+E fed WT mice, which was reduced by the depletion of *Camp* gene (Figure 3.7C). This indicated that the depletion of CRAMP also reduces other chemokines that may contribute to HFD+E-induced neutrophil infiltration in mice. It was showed previously

that feeding *Camp*^{-/-} mice with HFD+E significantly reduced the mRNA expression level of the chemokine receptor, CXCR-2, and that CRAMP may function as a CXCR-2 ligand in neutrophil chemotaxis. CXCL-1 is another well-defined CXCR-2 ligand, and we then evaluated whether the deletion of CRAMP affects the expression of CXCL-1 in HFD+E model. In WT mice, both acute ethanol and HFD feeding alone significantly increased the serum CXCL-1 concentration, and HFD+E treatment enhanced the induction of serum CXCL-1 compared to HFD feeding alone. However, this induction was not observed in *Camp*^{-/-} mice (Figure 3.7D). HFD+E induces a two-fold increase in CXCL-1 levels compared to HFD feeding alone in WT mice but not in *Camp*^{-/-} mice (Figure 3.7E). These data suggested that CRAMP acts as a chemoattractant for neutrophil infiltration induced by HFD+E in mice.

Figure 3.7 HFD+E treatment enhanced neutrophil clustering and chemokine expression in the livers of WT mice but not in *Camp*^{-/-} mice.

(A) CAE staining of paraffin embedded liver tissue. Infiltrated scattered neutrophils:

black arrows; clustered neutrophils: yellow arrow. (B) Quantified number of neutrophil

clusters (upper panel) and the area of formed clusters (lower panel) in the livers of HFD+E fed WT and *Camp*^{-/-} mice. (C) Hepatic mRNA levels of *Ly6G*, *Cxcr2-2* and *Mcp-1*. (D) Serum CXCL-1 concentration analyzed by ELISA. (E) Fold change of serum CXCL-1 comparing CD+E vs CD and HFD+E vs HFD in WT and *Camp*^{-/-} mice.

3.5 Discussion

Alcohol and obesity work synergistically in initiating and promoting alcohol associated liver disease (ALD) development. Obese alcoholics often have higher serum ALT levels and are more susceptible to progression from fatty liver to more advanced stages of ALD³⁶⁻⁴³. Oxidative stress and nitrosative damage were linked to the synergistic effects of obesity and alcohol consumption in rodents. Studies using genetically obese rats and mice associated exacerbated hepatic steatosis and injury with increased pro-inflammatory response and the impairment of hepatic lipid metabolism. A recent study by Chang et al. showed that feeding mice with short- or long-term HFD plus a single ethanol binge synergistically increased the serum and hepatic free fatty acids, chemokine CXCL-1 expression, and exaggerated neutrophil infiltration in the liver, eventually progressing to severe liver injury⁴⁵. This suggests that exacerbated neutrophil infiltration may contribute to HFD+E-induced liver injury.

In addition to anti-microbial potential¹⁶¹⁻¹⁶³, LL-37/CRAMP also has shown capacity to modulate inflammation and immunity^{87,164-167}. Zhang et al. demonstrated that LL-37 can act similarly to CXCR-2-specific chemokines CXCL1 in terms of receptor down-regulation and intracellular calcium mobilization on freshly isolated neutrophils. It

was hypothesized that the mouse cathelicidin, CRAMP, may play a role in HFD+E-induced liver injury, by exerting its chemoattracting activity.

10 weeks of HFD feeding significantly increased the body weight change in both WT and *Camp*^{-/-} mice over time, while more profound weight gain was found in WT mice. HFD fed WT mice consumed much more food than HFD fed *Camp*^{-/-} mice and had significantly higher body fat mass, reflected by the absolute weight of epididymal white adipose tissue. Additionally, WT mice fed with HFD had dramatically enlarged adipocytes in the eWAT tissue compared to *Camp*^{-/-} mice.

Adipocytes play an important role in energy and glucose metabolism. In addition to serving as a site for energy storage (in the form of triglycerides), adipocytes act as endocrine cells, secreting molecules that regulate energy expenditure, food intake, and glucose metabolism. Recent findings suggest that the size of adipocytes is a major modulator of their endocrine function¹⁷⁶. In studies of bacteria-induced skin infection, researchers observed rapid proliferation of preadipocytes and expansion of the dermal fat layer after infection of the skin by *Staphylococcus aureus*. This host defense function was mediated through the production of CRAMP from adipocytes since CRAMP production was decreased by inhibition of adipogenesis, and adipocytes from *Camp*^{-/-} mice lost the capacity to inhibit bacterial growth¹⁷⁷⁻¹⁷⁹. This interesting study suggested a protective role of adipose tissue in inflammation-related systemic disease, and that the increased fat body mass by HFD treatment in WT mice in the present study could serve as a protective mechanism against HFD-induced pro-inflammatory response, which is associated with an up-regulation of CRAMP, from the inflammation aspect. In contrast to what was shown in the present study, i.e., that lacking CRAMP suppress the weight gain of mice fed with

HFD, another study demonstrated that Lentiviral cathelicidin overexpression reduced the fat mass of HFD-treated diabetic mice through inhibition of hepatic fatty acid translocase (CD36)⁸⁸. This implied a distinct function of exogenous and endogenous CRAMP in regulating lipid metabolism under different disease conditions.

A significant difference in glucose tolerance between WT and *Camp*^{-/-} mice was not observed by the end of 10 week-HFD feeding period. However, HFD feeding significantly increased the fasting serum glucose level and induced glucose intolerance in *Camp*^{-/-} mice at an early stage but that was attenuated at a later stage; while HFD treatment increased fasting glucose levels and induced glucose intolerance over time in WT mice. It was further showed that *Camp*^{-/-} mice were protected from HFD+E-induced liver injury and hepatic fat accumulation, suggesting that the deletion of CRAMP desensitized the mice to HFD-induced glucose intolerance and HFD+E-induced liver injury.

Whether HFD+E treatment regulates CRAMP expression has not been reported yet. In the present study, the combination of HFD and alcohol consumption significantly increased CRAMP protein level in the liver, while the *Camp* mRNA was not changed by HFD+E. The induction of CRAMP was accompanied by significantly increased chemokine receptor CXCR-2, which plays a crucial role in neutrophil recruitment, suggesting that there is an enhanced neutrophil infiltration by HFD in the liver. Indeed, immunofluorescence staining of the neutrophil marker, Ly6G, revealed a much stronger positive staining in the livers of HFD+E fed WT mice compared to the CD+E fed controls, and interestingly, this was co-localized with increased hepatic CRAMP protein. Moreover, HFD+E treatment significantly decreased the protein level of CRAMP in the

spleens of WT mice, and surprisingly, spleen *Cxcr-2* mRNA level was significantly reduced by HFD+E treatment. This implied a potential organ-organ interaction between the liver and the spleen regarding CRAMP regulation.

Another interesting finding of this study is that CRAMP may involve in neutrophil clustering and thus, may enhance the pro-inflammatory response. HFD+E induced an increase of infiltrated neutrophils in both WT and *Camp*^{-/-} mice, but they were more scattered in the livers of *Camp*^{-/-} mice while being more clustered in WT mice. *Camp*^{-/-} mice had significantly decreased hepatic mRNA levels of the chemokines *Cxcl-2* and *Mcp-1*, which were dramatically induced by HFD+E in WT mice.

The clustering of neutrophils in the damaged sites is also referred to as neutrophil swarming¹⁸⁰, and a series of sequential phases have been described: (1) initial chemotaxis of individual neutrophils close to the damage; followed by (2) amplified chemotaxis of neutrophils from more distant interstitial regions; leading to (3) neutrophil clustering. In the third stage, swarming neutrophils can amplify their recruitment in a feed-forward manner through intercellular communication via leukotriene B4 (LTB4). The propagation of neutrophil recruitment leads to multiple, dense neutrophil cell clusters at the site of inflammation. Human cathelicidin LL-37 has been shown to induce LTB4 by human macrophages¹⁸¹ and LTB4, in turn, triggers the release of LL-37 from human neutrophils in innate immune responses¹⁸². This suggests that CRAMP may play a role in regulating neutrophil clustering through macrophage-derived LTB4.

Taken together, the studies in this chapter showed that *Camp*^{-/-} mice were desensitized to HFD-induced body weight gain and adipocyte enlargement, and deficiency of CRAMP protected mice from HFD+E-induced liver injury and steatosis.

Importantly, HFD+E treatment induced a dramatic increase in neutrophil infiltration and chemokine expression in the livers of WT mice, which was not observed in *Camp*^{-/-} mice. This indicated that CRAMP may act as a chemoattractant to enhance neutrophil infiltration induced by HFD+E treatment, causing exacerbated liver damage. In addition, significantly decreased mRNA levels of hepatic *Tgf-β* and *Col-1a1* were found in *Camp*^{-/-} mice compared to WT mice (data not shown), suggesting that CRAMP may play a role in hepatic stellate cells activation. More comprehensive and well-designed *in vitro* studies are needed to further confirm and elucidate the role of CRAMP in neutrophil regulation in different cell types in the liver.

CHAPTER 4 SUMMARY AND DISCUSSION

4.1 Cathelicidin (LL-37/CRAMP) and Its Role in Alcohol-associated Liver Disease

Antimicrobial peptides (AMPs) are short and generally positively charged peptides found in a variety of living organisms from bacteria to humans. AMPs were first identified and recognized as host defense peptides that can directly kill invading microorganisms. In addition to direct antimicrobial activity, AMPs carry immunomodulatory properties¹⁸³, which make them especially interesting compounds for the development of novel therapeutics. Several AMPs are currently being evaluated in clinical trials as novel anti-infectives, but also as new pharmacological agents to modulate the immune response, promote wound healing, and/or prevent post-surgical adhesions¹⁷⁰. The human gastrointestinal (GI) tract harbors a complex and dynamic population of microorganisms, the gut microbiota, which is now considered to be a hidden metabolic organ. Gut microbiota are crucially regulated by AMPs. The interdependence of the gut and the liver explains why disturbances in the intestinal barrier result in increased portal influx of bacteria or their products to the liver, where they cause or worsen a range of hepatic diseases/injury. The part played by intestinal microbes in liver diseases such as alcohol-associated liver disease (ALD) or bacterial infection in advanced liver disease has long been known¹⁷⁰. Previous studies have demonstrated critical role of AMPs in the pathogenesis of ALD^{61,76,77}. Reg3 lectins and

defensins were proved to be protective in ALD, through inhibition of pathogenic bacterial overgrowth and translocation of bacteria and toxic products into the liver^{76,78}. Moreover, overexpression of Reg3 lectin or synthetic human defensin attenuated alcohol-induced dysbiosis and liver injury.

In the present dissertation, the role of another important AMP, cathelicidin (known as LL-37 in humans and CRAMP in mice) in alcohol-associated liver disease was investigated in two different experimental models using WT and *Camp*^{-/-} mice. It was showed in **Chapter 2** that CRAMP deficiency exacerbated ALD with enhanced inflammasome activation as shown by elevated serum IL-1 β level. Although *Camp*^{-/-} mice had comparable serum endotoxin levels compared to WT mice after alcohol feeding, hepatic LPS binding protein and CD14 were increased. Serum levels of UA, a signal 2 molecule in inflammasome activation, were positively correlated with serum levels of IL-1 β in alcohol use disorder patients with ALD and were increased in *Camp*^{-/-} mice fed alcohol. These data provided strong evidence regarding inflammasome regulation by CRAMP in ALD. It was furtherly showed in *in vitro* studies that CRAMP peptide inhibited LPS binding to macrophages and inflammasome activation stimulated by a combination of LPS and UA, and that deficiency of CRAMP induced increased production of UA possibly via enhanced oxidative stress. One of the most important experimental strategies of this study is the use of gain-of-function approach as a treatment by exogenously giving back synthetic CRAMP peptide to alcohol-fed WT and *Camp*^{-/-} mice. CRAMP administration decreased serum UA and IL-1 β concentrations and rescued the liver from alcohol-induced damage in both WT and *Camp*^{-/-} mice.

Another mouse model of ALD was used in the study in **Chapter 3**. Long term HFD feeding plus binge alcohol gavage mimics acute ASH in obese binge drinkers and has great clinical implications. A recent study by Chang et al. showed that feeding mice with short- or long-term HFD plus a single ethanol binge synergistically increased the serum and hepatic free fatty acids, chemokine CXCL-1 expression and exaggerated neutrophil infiltration in the liver, eventually leading to severe liver injury ⁴⁵. This suggests that exacerbated neutrophil infiltration may contribute to HFD plus alcohol-induced liver injury. The finding that a single oral binge exposure of ethanol can trigger acute liver injury in the presence of short-term or long-term HFD feeding highlights the risk of even episodic binge drinking in obese/overweight individuals. LL-37/CRAMP were shown to act on freshly isolated neutrophils similarly to CXCR-2-specific chemokines (CXCL-1) in terms of receptor down-regulation and intracellular calcium mobilization ¹⁵⁸. In **Chapter 3**, *Camp*^{-/-} mice were desensitized to HFD-induced obesity and glucose intolerance; importantly, *Camp*^{-/-} mice were protected against HFD+E-induced liver injury, steatosis, neutrophil infiltration and pro-inflammatory chemokine expression. The study provided evidence that CRAMP functions in neutrophil trafficking regulation mainly through the well-characterized chemokine receptor CXCR-2, but not the other two receptors, FPR2 and P2X7, that have also been proposed as CRAMP receptors in macrophage regulation. This confirmed the notion that with HFD+E treatment, the immune regulatory activity of CRAMP leans more on the neutrophil chemoattracting aspect, but not on the TLR-4 mediated macrophage activation. Moreover, it was demonstrated in the present study that HFD+E induced increases in

infiltrated neutrophil number and cluster formation, and that Ly6G positive neutrophils colocalized with increased CRAMP protein in the livers of WT mice.

CRAMP is expressed in multiple organs and different cell types, and the present study showed that CRAMP is highly expressed in the spleen under physiological conditions. Analyzing the CRAMP levels in the spleen revealed that HFD+E treatment significantly decreased the protein level of CRAMP in the spleen of WT mice, and surprisingly, spleen CXCR2 mRNA expression was significantly reduced by HFD+E. This implied a potential organ-organ interaction between the liver and the spleen for CRAMP regulation. The most interesting finding of this study is CRAMP may involve in neutrophil clustering and thus may enhance the pro-inflammatory response. HFD+E induced an increase of infiltrated neutrophils in both WT and *Camp*^{-/-} mice, but they were more scattered in the livers of *Camp*^{-/-} mice while being more clustered in livers of WT mice. *Camp*^{-/-} mice also had significantly decreased hepatic mRNA levels of chemokines *Cxcl-2* and *Mcp-1*, which were dramatically induced by HFD+E in WT mice. This indicated that the depletion of CRAMP also reduces other chemokines that may contribute to HFD+E-induced neutrophil infiltration in mice.

A major question remains regarding the regulating activity of CRAMP in “inflammasome-inhibition” and “neutrophil-chemoattracting” in different disease models that were used in the present dissertation. CRAMP exacerbated binge alcohol-induced liver damage in chronic alcohol-primed mice, while attenuating binge alcohol-induced liver injury in HFD-primed mice. To try to answer this question, some of the key parameters in the two animal models were analyzed and compared side by side. HFD+E induced an increased *F4/80* mRNA expression in the livers of WT mice, but not in *Camp*^{-/-}

^{-/-} mice, suggesting that HFD+E enhanced macrophage activation is suppressed by the deletion of CRAMP (Figure 4.1A). HFD+E treatment did not have an impact on inflammasome-dependent *Il-1 β* and *Il-18 mRNA* expression in WT mice but significantly increased the anti-inflammatory cytokine *Il-10* mRNA level. In contrast, HFD+E treatment significantly increased *Il-1 β* and *Il-18 mRNA* expression with no change in *Il-10* mRNA expression in *Camp^{-/-}* mice, indicating an increased level of inflammasome activation by HFD+E. This confirmed our observations in the 24D+1B alcohol feeding model that CRAMP deficiency exacerbated alcohol-induced *Il-1 β* upregulation (Figure 4.1B). However, this induction of *Il-1 β* upregulation in *Camp^{-/-}* mice may only have minimal effects on the overall inflammatory response in HFD+E treated mice, as HFD+E induces significantly higher levels of chemokines compared to binge on chronic ethanol feeding, according to two recent studies^{45,184}. From the LPS signaling aspect, HFD+E significantly induced serum endotoxin in WT but not *Camp^{-/-}* mice, and HFD+E fed *Camp^{-/-}* mice had much lower serum endotoxin levels compared to HFD+E fed WT mice (Figure 4.1C). Surprisingly, mRNA expression of *Lbp* was decreased by HFD+E treatment in WT mice while it was increased in *Camp^{-/-}* mice. *Camp^{-/-}* mice had significantly higher *Lbp* expression (Figure 4.1D). LBP has a concentration-dependent dual role in the pathogenesis of gram-negative sepsis: low concentrations of LBP enhance the LPS-induced activation of mononuclear cells (MNC), whereas the acute-phase rise in LBP concentrations inhibits LPS-induced cellular stimulation¹⁸⁵. This suggested that in *Camp^{-/-}* mice, LPS-signaling might be inhibited by higher concentrations of LBP. Indeed, the mRNA expression of other two key molecules involved in LPS signaling, *Cd14* and *Tlr-4*, were decreased in *Camp^{-/-}* mice compared to WT mice fed with HFD+E (Figure

4.1D). The neutrophil infiltration in 24D+1B model in the livers of WT and *Camp*^{-/-} mice were analyzed as well, with the addition of two synthetic CRAMP peptide treatment groups: AF+CRAMP WT and AF+CRAMP *Camp*^{-/-}. Different from what was observed in the livers of WT mice fed HFD+E, no neutrophils clusters were detected in 24D+1B ethanol-fed WT mice. Importantly, exogenous CRAMP peptide increased neutrophil infiltration in the livers of 24D+1B ethanol-fed WT mice, which were decreased by CRAMP peptide treatment in the livers of AF fed *Camp*^{-/-} mice (Figure 4.1E). This again, confirmed the chemoattracting role of CRAMP on neutrophils in WT mice, and indicated that exogenous CRAMP works differentially in terms of neutrophil infiltration under different disease conditions.

Figure 4.1 Inflammasome activation in HFD+E model and CAE staining in 24D+1B model evaluation.

(A) Hepatic *F4/80* mRNA. (B) Hepatic *Il-1 β* , *Il-18* and *Il-10*. (C) Serum endotoxin level. (D) Hepatic mRNA expression of *Lbp*, *Cd14*, *Tlr-4*. (E) CAE staining of liver tissue. Black arrow: infiltrated neutrophils. Data are expressed as mean $\pm$ SEM, (n $\geq$ 5). *p<0.05, **p<0.01.

4.2 Strengths and Weaknesses

The studies in this dissertation explored the dual role of the antimicrobial peptide, CRAMP, in two different alcohol-associated liver disease models. The role of CRAMP is somewhat confounding, as it exhibits both pro-and anti-inflammatory activity. A major obstacle in the study of cathelicidins is the inability of exogenous LL-37 or CRAMP to mimic the activity of their endogenous counterparts. In Chapter 2, using a loss- and gain-of function approach, we dissected the role of CRAMP in alcohol-induced liver injury in a 24 days chronic alcohol feeding plus one binge alcohol gavage model (24D+1B). CRAMP deficiency exacerbated, while exogenous CRAMP peptide alleviated, alcohol-induced liver injury through inhibition of inflammasome activation by decreasing LPS function and uric acid (UA) production. The majority of the studies investigating the role of AMPs in ALD have been focused on the inhibition of pathogenic bacteria growth, and the inhibition of translocation of bacteria and toxic products from gut lumen to the liver. CRAMP deficiency induced gut microbiota dysbiosis, but more importantly, it was clearly demonstrated that CRAMP not only affects the gut microbiota homeostasis, but also participates in immune regulation. *Camp*^{-/-} mice had increased levels of serum IL-1 β , which is accompanied by the enhancement of LPS signaling and increased levels of uric acid production, both of which are critical signals required by NLRP3-dependent

inflammasome activation. Furthermore, the *in vitro* study showed that the deficiency of CRAMP induces UA production, possibly through exacerbated oxidative stress in the livers in ALD. Another strength of the study in Chapter 2 is the inclusion of human study. Analysis of the banked serum and liver biopsy samples from patients with ALD confirmed the observations in animal study and demonstrated that human cathelicidin, LL-37, is dysregulated in ALD and there is a positive correlation between serum IL-1 β and serum UA concentrations.

The animal model in Chapter 3 simulated acute alcohol-induced liver damage in obese binge drinkers. *Camp*^{-/-} mice are protected from HFD+E-induced liver injury, and this is possibly due to the deficiency of CRAMP, which leads to reduced body weight gain and food intake. Importantly, the impairment of neutrophil infiltration under HFD+E stimulation. *Camp*^{-/-} mice are also desensitized to HFD-induced glucose intolerance over time, suggesting other mechanisms exist in addition to the chemoattracting activity of CRAMP. It could not be ignored that CRAMP may directly affect lipid metabolism and gut barrier function, as the more pronounced enlargement of adipocytes were seen in WT mice but not in *Camp*^{-/-} mice. WT mice also had increased levels of serum endotoxin, which were not seen in *Camp*^{-/-} mice.

Another concern is the unique expression pattern of CRAMP in different organs and cell types. It was shown in the 24D+1B model that CRAMP is highly expressed in the spleen and eWAT under physiological conditions, and CRAMP is upregulated by alcohol consumption at the mRNA and protein levels. However, it is not clear where this accumulated CRAMP protein in the liver comes from. In Chapter 3, IF staining revealed a colocalization of CRAMP and Ly6G-positive neutrophils in the livers of WT mice by

HFD+E treatment, but this is not exclusive. CRAMP acts as a chemoattractant to recruit neutrophils, however, the neutrophils themselves produce a lot of CRAMP upon stimulation. It is still an open question as to whether CRAMP is expressed mainly in the liver by hepatocytes or by the resident macrophages (Kupffer cells), by the infiltrated neutrophils, or whether CRAMP traveled as a hormone that was originally secreted by other organs, such as the spleen, upon alcohol exposure.

Another weakness of this study is the limited sample size of human biopsies. The discrepancies of cathelicidin regulation in ALD patients and healthy controls could be attributed to the small sample size ^{186,187}.

In summary, the dual role of CRAMP in alcohol-associated liver disease have been explored in two experimental models of ALD. CRAMP plays a protective role in binge alcohol-induced liver injury in chronic alcohol-primed liver, through the inhibition of LPS and UA-induced inflammasome activation. However, CRAMP acts as a chemoattractant in HFD-primed liver to enhance the recruitment and aid in the trafficking and activation of neutrophils, which makes the liver prone to binge alcohol-induced liver damage as a second hit. The divergent effects of CRAMP in different ALD models revealed in this dissertation provide a better understanding of the complex roles of cathelicidin in alcohol-associated liver disease. With the presence of obesity as a comorbidity for the disease progression, the activity of cathelicidin is modulated by complex interactions with the microenvironment as well as with the disease background. The inhibitory effects on inflammasome activity and the enhancement on neutrophil infiltration in different disease models by CRAMP emphasized the importance of the

critical balance between anti- and pro-inflammatory responses upon tissue damage and that the role of CRAMP in alcoholic liver disease is dependent on the disease status.

4.3 Future Directions

4.3.1 Further Validation of the Inhibitory Effects of CRAMP on LPS Signaling and Uric Acid Production and Metabolism

CRAMP deficiency exacerbated alcohol-induced liver injury through inhibition of inflammasome priming and activation signals. Deletion of CRAMP led to increased LPS signaling protein and increased serum UA in alcohol fed mice, and serum UA levels were positively correlated with IL-1 β concentrations in ALD patients. Although it was shown in an *in vitro* study that CRAMP, indeed, inhibits LPS binding to macrophages in a dose-dependent manner, the *in vivo* evidence is still lacking. Immunoprecipitation assay could be done using serum and liver lysates samples from alcohol fed WT and *Camp*^{-/-} mice, to examine the interactions of CRAMP between LPS and its signaling proteins *in vivo*. Additionally, the mechanisms through which CRAMP regulates UA production have not been completely addressed in this dissertation. The present study showed an increased ROS formation in the livers of *Camp*^{-/-} mice, and that hepatocytes transfected with *Camp*-targeting siRNA produced more UA in the supernatant than non-targeting siRNA-transfected hepatocyte, indicating that CRAMP might regulate UA production by the hepatocytes through the induction of oxidative stress. But other cell types in the liver, such as Kupffer cells, also produce uric acid following alcohol treatment. The effects of CRAMP on UA regulation in different cell types should be explored.

4.3.2 Evaluation of Lipid Metabolism Regulation by CRAMP

In 24D+1B model, synthetic CRAMP peptide treatment almost completely reversed hepatic fat accumulation in alcohol fed-WT and *Camp*^{-/-} mice. In HFD+E feeding model, a drastic reduction of liver steatosis and adipocyte enlargement was observed in *Camp*^{-/-} mice. While the present studies focused on the inflammasome activation and neutrophil infiltration, it is worth noting that CRAMP may have a direct impact on lipid metabolism in the liver and adipose tissue. Analysis on the fatty acid synthesis, oxidation and transportation could be conducted to elucidate the potential role of CRAMP on lipid metabolism in mice under physiological conditions and under binge alcohol challenged conditions in chronic ethanol or long term HFD-fed mice.

4.3.3 Determine the Effects of CRAMP on Gut Barrier Function

Binge on chronic alcohol-fed *Camp*^{-/-} mice had comparable serum endotoxin levels compared to their WT controls, while binge alcohol in HFD-fed *Camp*^{-/-} mice produced significantly lower levels of serum endotoxin than in WT mice. This implies a distinct regulation pattern of CRAMP on gut barrier function under different disease status. Tight junction proteins and permeability assays can be performed to examine the role of CRAMP in regulating gut barrier function in different ALD models.

4.3.4 Further Evaluation of Neutrophil Infiltration Regulation by CRAMP through Chemokine Receptor CXCR-2

Additional *in vitro* study is still needed to further confirm the hypothesis that CRAMP acts through the chemokine receptor, CXCR2, to recruit neutrophils in HFD+E

feeding model. *Cxcr-2*^{-/-} mice could be used in the animal model applied in Chapter 3, to see whether HFD+E induces CRAMP expression in *Cxcr-2*^{-/-} mice, and whether neutrophil infiltration is suppressed in *Cxcr-2*^{-/-} mice due to the loss of receptor that CRAMP acts through. Another *in vitro* study could be designed using synthetic CRAMP peptide-treated neutrophils isolated from WT and *Cxcr-2*^{-/-} mice, to determine if CRAMP peptide treatment increases neutrophil migration through the induction of CXCR-2 by the treatment of fatty acids and ethanol.

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APPENDIX

Appendix 1 List of Abbreviations

24D+1B	24 days of ethanol feeding plus 1 binge ethanol gavage
AAH	Acute alcoholic hepatitis
ADH	Alcohol dehydrogenase
AF	Alcohol fed
ALD	Alcoholic liver disease
ALDH2	Aldehyde dehydrogenase
ALT	Alanine aminotransferase
AMP	Antimicrobial peptides
ASC	Apoptosis-associated speck protein with a caspase activation and recruitment domain
ASH	Alcohol-associated steatohepatitis
AST	Aspartate aminotransferase
ATP	Adenosine 5'-triphosphate
AUD	Alcohol use disorder
BCA	Bicinchoninic acid assay
BHI	Brain heart infusion
BMDM	Bone marrow-derived macrophages
BSA	Bovine serum albumin
BW	Body weight
CAE	Chloracetate esterase
CD	Control diet
CD14	Cluster of differentiation 14

CD73	Cluster of differentiation 73
CRAMP	Cathelicidin-related antimicrobial peptide
CV	Central vein
CXCL	Chemokine (C-X-C motif) ligand
CYP2E1	Cytochrome P450 2E1
DAMPs	Danger-associated molecular patterns
DFO	Desferrioxamine
DHE	Dihydroethidium
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DSS	Dextran sulfate sodium
<i>E. coli</i>	<i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
EtOH	Ethanol
eWAT	Epididymal white adipose tissue
FAEE	Fatty acid ethyl esters
FBS	Fetal Bovine Serum
FFA	Free fatty acid
FITC	Fluorescein isothiocyanate
FPR1/FPR2	Formyl peptide receptor-like 1 / 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H&E	Hematoxylin and eosin
H ₂ O ₂	Hydrogen peroxide
HC	Healthy controls
HCC	Hepatocellular carcinoma
HFD	Hight fat diet
HIF1 α	Hypoxia inducible factor 1 α
HRP	Horseradish peroxidase
I.P.	Intraperitoneally

IBD	Inflammatory bowel disease
ICAM-1	Intercellular adhesion molecule 1
IF	Immunofluorescence
IL-	Interleukin-
KO	Knockout
LAL	Limulus amebocyte lysate
LB	Lysogeny broth
LBP	LPS binding protein
LGG	<i>Lactobacillus rhamnosus</i> GG
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
LTDH	Lifetime drinking history
Ly6G	Lymphocyte antigen 6 complex locus G6D
M-CSF	Macrophage colony-stimulating factor
MELD	Model for end-stage liver disease
MEM	Minimum essential media
MNC	Mononuclear cells
mRNA	Messenger ribonucleic acid
MRS	De Man, Rogosa and Sharpe agar
NADH	Nicotinamide adenine dinucleotide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIAAA	National Institute on Alcohol Abuse and Alcoholism
NLRP3	NLR family pyrin domain containing 3
NT	Non-targeting
OCT	Optimal cutting temperature compound
OGTT	Oral glucose tolerance test
OTU	Operational Taxonomic Units
P2X7	P2X purinoceptor 7

PAMPs	Pathogen-associated molecular patterns
PF	Pair fed
PT	Portal tract
qRT-PCR	Quantitative real time polymerase chain reaction
REG3B or REG3G	Antimicrobial-regenerating islet-derived (REG)-3 lectins B or G
RIPA	Radioimmunoprecipitation assay
RNAse	Ribonuclease
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SCFA	Short chain fatty acid
SDS-PAGE	Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis
SEM	Standard error of the mean
TBST	Tris-buffered saline/Tween 20
TG	Triglyceride
TLFB	Timeline follow back
TLRs	Toll-like receptors
TM7	Candidatus bacteria
TNF- α	Tumor necrosis factor α
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
UA	Uric acid
VCAM-1	Vascular cell adhesion molecule 1
VDR	Vitamin D receptor
WT	Wild type
XO	Xanthine oxidase

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EDUCATION

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RESEARCH EXPERIENCE

PhD Candidate, University of Louisville, 2016-Present

Proposed Dissertation Title: Antimicrobial Peptide Cathelicidin Protects from Alcohol-Induced Liver Injury in Mice Through Modulating Gut Microbiota and Inhibiting Inflammasome Activation.

- Responsible for experiments design and performance, including mouse husbandry, genotyping, special diet feeding, tissue samples harvesting, process and analysis *in vitro* and *ex-vivo* experiments using primary cells and specific cell lines.
- Responsible for data analysis, manuscript drafting and revision, research grant writing.
- Mentor: Dr. Wenke Feng, Associate Professor of Medicine, University of Louisville.

Research Assistant, University of Louisville, 2013-2015

Project 1: Investigating the role of duodenal iron transporters in alcoholic cirrhosis (AC) patients with hepatic iron overload.

- Responsible for experimental design and performance including human duodenum biopsy slides preparation and analysis, serum samples preparation and analysis.
- Perform data analysis and draft writing & revision of manuscript

Project 2: Testing the hypothesis that intestinal hypoxia inducible factor (HIF)-1 α deletion exacerbates alcoholic liver disease (ALD) and exploring the underlying mechanisms.

- Actively participated and contributed to the initial stage of the project, responsible for mouse husbandry, genotyping and alcohol feeding.
- Supervised by: Dr. Wenke Feng, Associate Professor of Medicine, University of Louisville.

Research assistant, Northwest University, China, 2012-2013

Project 1: Investigating the role of flagellum gene *FlgE* in antibiotic resistance of *Pseudomonas Aeruginosa* (PA)

Project 2: Exploring the transcriptional mechanisms of type III secretion system in *Pseudomonas aeruginosa*

Project 3: Examine the inhibition on pathogenicity of *Pseudomonas aeruginosa* by baicalin and its mechanisms in regulating the swarming motility and biofilm formation.

- As part of the research team, participated in the construction of the flagellum gene *FlgE* Double Site knockout mutant of *Pseudomonas Aeruginosa* PAO1; performed the bacterial motility experiment and the construction of transposon mutagenesis involved in type III secretion system. Engaged in the construction of fruit fly infection model and data analysis.
- Supervisor: Dr. Kangmin Duan, Professor of College of Life Sciences, Northwest University.

TEACHING EXPERIENCE

Pharmacology & Toxicology Graduate Program: Student-oriented courses, 2016-2017

1. Pharmacology I&II: Goodman & Gilman's 12th Edition:

- The Pharmacological Basis of Therapeutics: Drug toxicity and poisoning; Adrenergic agonists & antagonists; Treatment of central nervous system degenerative disorders; Targeted therapies: Tyrosine kinase inhibitors & monoclonal antibodies; Anti-arrhythmic drugs; Treatment for disorders of GI motility and water flux.

2. Toxicology I&II: Casarett & Doull's Toxicology- The Basic Science of Poisons, 8th Edition:

- Respirative toxicity; Chemical carcinogens; Epidemiologic studies & agencies in determining carcinogen; Reproductive toxicology; Environmental toxicants: PCBs.

ADMINISTRATION EXPERIENCE

LinkedIn moderator & E-bulletin Writer, International Society for Biomedical Research on Alcoholism (ISBRA) Communication Committee, 2019-present

- Support and manage ISBRA Communication Committee LinkedIn account. Also responsible for research news, job posting and corporation opportunities relevant to research on alcoholism.
- Collect, organize, edit and write the section of news and events relevant to research on alcoholism nationally and internationally. Also participate in the review, proof reading, editing and formatting on overall newsletters.

HONORS & AWARDS

- Student Merit Award for Research Society on Alcoholism (RSA) meeting, 2019
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- Winner of student poster award (First place) in American Society for Pharmacology and Experimental Therapeutics (ASPET) of Experimental Biology (EB) meeting, 2018
- Student Merit Award for Research Society on Alcoholism (RSA) meeting, 2018
- Winner of Annual Research Louisville Symposium (Second place), 2017
- Winner of Northwest University Students' Research Symposiums (First place), 2015

PROFESSIONAL SOCIETY

- Invited member of International Society for Biomedical Research on Alcoholism (ISBRA), 2019-present
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- Member of Research Society on Alcoholism (RSA), 2018-present

PUBLICATIONS

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