Innate Immunity As Mediator of Cell Death and Inflammation in Alcoholic Liver Disease

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INNATE IMMUNITY AS MEDIATOR OF CELL DEATH AND INFLAMMATION
IN ALCOHOLIC LIVER DISEASE

A Dissertation Presented

By

Arvin Iracheta-Vellve

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
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November, 1, 2017

Translational Science
DISSERTATION EXAMINATION COMMITTEE

INNATE IMMUNITY AS MEDIATOR OF CELL DEATH AND INFLAMMATION IN ALCOHOLIC LIVER DISEASE

A Dissertation Presented
By
Arvin Iracheta-Vellve

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November 1, 2017
EPIGRAPH

To dare is to lose one's footing momentarily.

Not to dare is to lose oneself.”

— Søren Kierkegaard
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ABSTRACT

Central driving forces in the pathogenesis of liver disease are hepatocyte death and immune cell-driven inflammation. The interplay between outcomes, stemming from these two major cell types, is present from the earliest ethanol exposure, and are both determinants in advanced stages of liver disease particularly in alcoholic liver disease (ALD). The complexities associated with advanced ALD are many and therapies are limited. Due to the liver's role in ethanol metabolism and filtering gut-derived products, it is becoming increasingly clear that innate immunity plays a central role in triggering activation of cell death and inflammatory pathways in ALD. We identified interferon regulatory factor 3 (IRF3) activation as a mediator of hepatocyte death as the first event after ethanol exposure, and the inflammasome as a protein complex responsible for the subsequent inflammatory cascade, driven by the NLRP3 inflammasome.

Our novel findings in murine samples and human patients with alcoholic hepatitis demonstrate that ethanol-induced inflammasome activity results in Caspase-1-mediated pyroptosis and extracellular ASC aggregates in the liver and circulation. Pyroptosis can be abrogated by therapeutic inhibition of inflammasome components, NLRP3 or Caspase-1. Taken together, the event leading to mtDNA release into the cytoplasm is the inception of the pathogenesis of ALD, triggering hepatocyte death, culminating in a pro-inflammatory cascade driven by the NLRP3 inflammasome and pyroptotic release of ASC.
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LIST OF ABBREVIATIONS

Acetaminophen (APAP); alcoholic hepatitis (AH); Alcoholic liver disease (ALD); 2-aminoethoxydiphenyl borate (2APB); apoptosis-associated speck-like protein containing a CARD (ASC); bile acid (BA); carbon-tetrachloride (CCl₄); bone marrow (BM); Caspase (Casp); connexin (Cx); cryopyrin-associated periodic syndromes (CAPS); cyclic GMP-AMP (cGAMP); cGAMP synthase (cGAS); cytochrome C oxidase (COX); cytochrome P450-2E1 (CYP2E1); damage-associated molecular patterns (DAMPs); Defeat Alcoholic Steatohepatitis (DASH); diguanylate cyclase (DGC); double stranded (ds); endoplasmic reticulum (ER); ER stress inhibitors (ERSI); ethanol (EtOH); farnesoid X receptor (FXR); fatty acid synthase (FASN); gasdermin D (Gsdm D); hepatic stellate cells (HSC); 8-Hydroxy-2-deoxy-guanosine (8-OHdG); interferon (IFN); IFN receptor (IFNAR1); interferon regulatory factor 3 (IRF3); interleukin (IL); IL-1R antagonist (IL-1Ra); intracellular uricase (intUOX); Kupffer cells (KC); lipopolysaccharide (LPS); liver mononuclear cells (LMNCs); liver receptor homolog 1 (LRH-1); monosodium urate crystals (MSU); myeloperoxidase (MPO); NADH dehydrogenase subunit 6 (ND6); NOD-like receptors (NLRs); non-alcoholic steatohepatitis (NASH); obeticholic acid (OCA); pair-fed (PF); pathogen-associated molecular patterns (PAMPs); 4-phenylbutyrate (4PBA); Poly(ADP-ribose) polymerase (PARP); primary biliary cholangitis (PBC); protein kinase A (PKA); regulated IRE1-dependent decay of mRNA (RIDD); small heterodimer partner 1 (SHP-1); spleen tyrosine kinase (SYK); spliced Xbp1 (sXbp1); sterol
regulatory element–binding protein (SREBP); stimulator of interferon genes (STING); pattern-recognition receptors (PPRs); secreted uricase (ssUOX); TANK-binding kinase 1 (TBK1); tauroursodeoxycholic acid (TUDCA); Toll-like receptor 4 (TLR4); Tata-binding protein (TBP); transgenic (Tg); transmembrane G protein-coupled receptor 5 (TGR5); transmembrane protein 173 (Tmem173); tumor necrosis factor-alpha (TNF-α); unfolded protein response (UPR);
LIST OF RELEVANT PUBLICATIONS


PREFACE

Data for some figures in these chapters were generated in collaboration with members of the Szabo Lab. Some of our early work was published previously, and served as the basis for subsequent study. In particular, the identification of STING and IRF3 in mediating ALD from Chapter 2 was previously published in Petrasek J et al., PNAS, 2012. Data from Figure 2.3 was generated by Jan Petrasek; data generated in Figures 2.1, 2.2, 2.4(A-C), 2.5, 2.6, 2.7, 2.8, 2.9, 2.10 was generated with Jan Petrasek; study design and mouse experiment for data generated from Figure 2.11 was done with Benedek Gyongyosi; ddPCR assay from Figure 2.15 was performed with assistance from Abhishek Satishchandran.

Data on identification of ATP and uric acid as hepatocyte-derived DAMPs in ALD from Chapter 3 was previously published in Petrasek J et al., Journal of Leukocyte Biology 2015. To wit, the mouse experiments from Figure 3.1, 3.4, 3.8, 3.9, 3.10, 3.11 and 3.12 were performed by Jan Petrasek, with histological and biochemical analysis performed by Arvin Iracheta-Vellve; data generated in Figures 3.2, 3.3, 3.5, 3.6 were performed with Jan Petrasek; data generated in Figure 3.12 was performed by Banishree Saha; data analysis in Figure 3.13 was performed by Benedek Gyongyosi; data generated in Figures 3.15 and 3.19D was performed with Patrick Lowe; data generated in Figure 3.16 was performed with Charles D. Calenda; the mouse experiment for the basis of Figure 3.19A-C was performed with Aditya Ambade, with analysis done by me. Data on the role
SYK in activation of inflammasome signaling in ALD from Chapter 3 was previously published in *Bukong T et al., Hepatology 2016*. The mouse experiment for Figure 3.22 was performed with Terence Bukong. Data generated from Figure 3.22A, C, and D were generated by Terence Bukong, and Figure 3.22B generated by me.

Data generated from Chapter 4 on the role of bile acid receptor agonists in liver disease was performed with collaboration of Intercept Pharmaceuticals. The mouse experiments were performed by Jan Petrasek with assistance from Abhishek Satishchandran. Biochemical and histological analysis was performed by me with assistance from Charles Calenda.

Unless specifically highlighted above, all data and schematics were generated by me.
CHAPTER ONE: LIVER DISEASE AND INNATE IMMUNITY

Alcoholic liver disease

Disease spectrum and progression

Alcohol consumption is a common event in the western world. Alcohol abuse or dependence, however, is responsible for 40 to 50% of all liver-related deaths\(^1\). Alcoholic liver disease (ALD) is used to describe the full spectrum of alcohol-related pathology. The spectrum of ALD can be classified into three categories, steatohepatitis, alcoholic hepatitis (AH), and cirrhosis\(^2\). Some patients with steatohepatitis progress to fibrosis, where an inflamed liver in which dead hepatocytes are replaced by deposition of extracellular matrix proteins, altering the liver’s functional ability\(^3\). Advanced fibrosis results in cirrhosis and often end-stage liver failure. AH has a high mortality, associated with poor four-year survival especially in the presence of cirrhosis, ranging from 35% to 60 %. Due to the complexities of the disease, and the spectrum of severity displayed by patients with alcoholic liver disease, aspects of which remain poorly understood, there is still no FDA approved treatment for this disease. Corticosteroids are most commonly prescribed\(^4\), yet even these patients have a six-month mortality of 40%. These poor clinical outcomes highlight the urgent need to gain a better understanding of mechanism and therapeutic targets.
Figure 1.1 Spectrum of alcoholic liver disease

Alcoholic liver disease is a spectrum of various ethanol-induced pathologies. Upon initial ethanol exposure, hepatocyte death and steatosis. Inflammation stems, in part, due to microbial products entering the liver through 'leaky gut', activating the immune system. Subsequent and repeated ethanol exposure can result in alcoholic steatohepatitis, alcoholic hepatitis, fibrosis and even cirrhosis.
Ethanol

- Hepatocyte death
- Steatosis
- Steatohepatitis
- Fibrosis
- Cirrhosis

LPS
leaky gut

ethanol

Liver

hepatocyte
Kupffer cell
**Disease biology**

The complexity of ALD in its various stages is due to the involvement and dysfunction of multiple cell types and multiple organs. Although this work is focused entirely on the effects of ethanol on liver biology, ethanol consumption can have lasting effects on the brain, gut and microbiome. Within the liver, hepatocytes are responsible for processing of ethanol, opening the gates for metabolic imbalance that results in lipid accumulation in the liver, or steatosis. Further ethanol exposure results in the onset of inflammation, resulting in steatohepatitis. Inflammation itself can further increase steatotic state, recruiting more inflammatory cells, thus further escalating the disease and its severity. In this way, ethanol metabolism, directly and indirectly, damages hepatocytes and pushes inflammation through various innate immune pathways and crosstalk from cell-to-cell.

Accumulation of ethanol-induced oxidative stress is a key mechanism for hepatocyte injury. Hepatocytes are well equipped in neutralizing reactive oxygen species through production of glutathione among others. However, under chronic alcohol consumption, stores of glutathione are depleted, severely limiting a hepatocyte’s ability to buffer against ROS. In the liver, ethanol is mainly metabolized via its oxidation by alcohol dehydrogenase, yielding acetaldehyde. Acetaldehyde, its byproduct, is a highly toxic chemical. Roughly ten percent of the ethanol is metabolized by cytochrome P450-2E1 (CYP2E1) in microsomes. In this scenario, ROS and other ethanol intermediate metabolites are
produced, such as hydrogen peroxide and superoxide\textsuperscript{13-15}, forming protein and DNA adducts, lead to an increase in lipid peroxidation\textsuperscript{5,16} and even create carcinogenic products\textsuperscript{17}. The ethanol-inducible enzyme CYP2E1, expressed almost exclusively in zone 3 of the hepatic lobule, is used when alcohol dehydrogenase is saturated, thereby increasing oxygen demand and creating a hypoxic and ROS-susceptible microenvironment in the liver \textsuperscript{18}. The zonal distribution of CYP2E1 inversely correlates with oxygen gradient. In contrast, zone 1 near the hepatic artery, is oxygen-rich with low CYP2E1 expression\textsuperscript{19-21}. This hepatocyte heterogeneity throughout the hepatic lobule results in a zonal distribution of liver injury from metabolism of ethanol and other xenobiotics like carbon tetrachloride and acetaminophen. This zonal distribution of liver injury may explain why some hepatocytes are prone to undergo apoptosis and/or necrosis. As explained later, hepatic gap junctions may also play a role in this phenomenon.

**Innate immunity overview**

**TLR4 signaling**

Alcohol exposure creates intestinal permeability. This “leaky gut” allows bacterial lipopolysaccharide (LPS) wall fragments, also referred to as endotoxin, to reach the liver, an important line of defense against infection from the gut. Translocation of LPS to the liver triggers the innate immune system: Kupffer cells
(KC), the resident macrophages in the liver, recognize LPS. KC-recognition of LPS requires the Toll-like receptor 4 (TLR4), which triggers two downstream signaling pathways. The TLR4/MyD88 pathway activates a network of cytokines, such as tumor necrosis factor-alpha (TNF-α), resulting in liver inflammation\textsuperscript{22}. The TLR4/TRAM/TRIF pathway, conversely, triggers TANK-binding kinase 1 (TBK1)-mediated interferon regulatory factor 3 (IRF3) activation\textsuperscript{23}. The essential role of the TLR4 signaling in ALD was demonstrated in mice lacking functional TLR4 that showed attenuation of alcoholic steatohepatitis\textsuperscript{24,25}. 
Figure 1.2 TLR4 activation of TBK1 and IRF3

TLR4 can be activated by gut-derived LPS. This simplified model shows the MyD88-independent signaling via its adaptors TRAM and TRIF. Signal transduction leads to activation of TBK1, the kinase responsible for IRF3 phosphorylation and activation.
In previous studies, our lab reported that the MyD88-dependent pathway was dispensable for ALD, and observed complete protection from alcohol-induced inflammation, steatosis and injury in mice deficient in IRF3\textsuperscript{26}, suggesting that the pathogenic effects of TLR4 in ALD were mediated via the TRAM/TRIF-dependent pathway. However, the mechanisms by which IRF3 causes ALD remain obscure.

**IRF3 signaling**

IRF3 is a transcription factor that plays an important and well described role in response to viral infection\textsuperscript{27-31}. IRF3 is in its active state when phosphorylated and dimerized. The eventual nuclear translocation of activated IRF3 leads to the production of type-I interferon (IFN) and many IFN-inducible genes that create an anti-viral state. Traditionally, IRF3 activation has been shown to occur via TLR signaling\textsuperscript{32}. However, stimulator of interferon genes (STING)-mediated activation of IRF3 is independent of TLR signaling. Moreover, IRF3 has other roles in addition to its function as a transcription factor: IRF3 can promote apoptosis in virus-infected cells\textsuperscript{33-35}. IRF3 associates with mitochondria and interacts with the pro-apoptotic protein BAX, leading to cytochrome c release and a caspase activation cascade. STING, previously referred to as transmembrane protein 173 (Tmem173), is an endoplasmic reticulum (ER) trans-membrane protein that acts as a secondary sensor to cytosolic double stranded (ds) DNA.
Activation of STING leads to a conformational change, which allows for TBK1 kinase recruitment to activate IRF3\textsuperscript{29}. STING activation of IRF3 occurs from intracellular double stranded (ds) DNA, from self or pathogen, in a sequence-independent manner\textsuperscript{36}.

There are several known intracellular dsDNA sensors that activate STING signaling in response to microbial infection\textsuperscript{37}. The ds DNA sensor cyclic GMP-AMP (cGAMP) synthase (cGAS)\textsuperscript{38-40}, is a known activator of STING by its synthesis of the cyclic dinucleotide 2’3’-cGAMP. A different type of cyclic dinucleotide synthesized by bacteria, c-di-GMP, is another strong STING activator\textsuperscript{41,42}. The bacterial cyclic dinucleotides are synthesized by diguanylate cyclase (DGC), an enzyme present in roughly 90% of bacteria\textsuperscript{43,44}. Cyclic dinucleotides are hydrolyzed by a variety of phosphodiesterases, converting cyclic dinucleotides to GMP. The potential role of cyclic dinucleotides in ALD is yet to be explored.
Figure 1.3 Endogenous and bacterial cyclic dinucleotide activation of STING

STING can be activated by cyclic dinucleotides synthesized by endogenous cGAS, which secretes 2′3′-cGAMP, or bacterial diguanylate cyclase, which secrete c-di-GMP. We hypothesize cGAS activation in hepatocytes results in 2′3′-cGAMP-mediated activation of STING. We also postulate that ethanol-induced ‘leaky gut’ results in STING and IRF3 activation in the liver through microbial products like LPS and c-di-GMP.
Hepatic gap junctions

A gap junction is formed by a pairing of head-to-head hemi-channels assembled on adjacent cells\textsuperscript{45}. These channels are made of connexin (Cx) proteins. The nomenclature for this Cx family of proteins is based on their respective molecular weights. In the liver, the majority of non-parenchymal cells express Cx43, whereas hepatocytes express ninety-percent of all Cx32 hepatic expression\textsuperscript{46}.

In the event of an infection, secretion of antiviral and pro-inflammatory cytokines to establish an antiviral state, such as IFNβ and TNFα, may not be enough. Viruses have evolved strategies for limiting secretion of these cytokines to evade host innate immunity\textsuperscript{47}. Gap junctions, by directly connecting cytosol from adjacent cells, allow for diffusion of ions, second messengers and metabolites\textsuperscript{48}. However, this diffusion of information is not cargo selective and not context-specific.

Even though pro-inflammatory signaling is powerful and essential in the event of an infection, it must be tightly regulated. Unregulated inflammation can lead to disease. A study showed that during acetaminophen overdose, an example of a sterile injury, gap junctions are responsible for the amplification of acute pro-inflammatory signaling induced by hepatocyte damage\textsuperscript{49}. Inhibition of gap-junction communication limited the extent of the injury and protected against liver failure \textit{in vivo}. Interestingly, in response to viral infection, cyclic dinucleotides were shown to spread through gap junctions\textsuperscript{50}. More specifically, gap junctions
allow for the spread of DNA-mediated cell-to-cell activation of IRF3\textsuperscript{51}. Cyclic dinucleotide communication through gap junctions triggers STING and IRF3 activation in neighboring cells, thus amplifying the anti-microbial response. Given the zonal distribution of hepatocyte damage in alcoholic liver disease, we hypothesize that activation of IRF3 via gap junction-mediated cyclic dinucleotide contributes to amplification of cell death and inflammation in ALD.
Figure 1.4 Proposed propagation of cyclic dinucleotides through hepatic gap junctions in ALD

Gap junctions allow the cell-to-cell transfer of cyclic nucleotides synthesized by cGAS. This leads to an amplification of STING activation to neighboring cells, and a pro-apoptotic and pro-inflammatory signaling cascade.
Programmed cell death

Cell death can present itself in a variety of ways. Necrosis and osmotic lysis are examples of unregulated, un-programmed cell death, resulting in LDH and DAMP release, and propidium iodide-positive staining. Programmed cell death depends on signal transduction pathways that eventually result in cell death. Apoptosis is a regulated, programmed cell death that results in ATP depletion, Annexin V-positive staining, and is ‘clean’ in nature, with no DAMP release – usually terminated by phagocytosis\textsuperscript{52,53}. In the event that phagocytosis does not remove cell debris, secondary necrosis (also commonly referred to as late apoptosis) will ensue\textsuperscript{54}. On the other hand, inflammasome-mediated pyroptosis and RIP1-mediated necroptosis are two forms of programmed cell death which result in a necrotic form of cell death\textsuperscript{55-57}.

Apoptosis in hepatocytes results from displacement or down-regulation of the Bcl-2 family of anti-apoptotic proteins and subsequent activation of pro-apoptotic BH3-only proteins such as BAX and BAK\textsuperscript{58-61}. These events lead to oligomerization of BAX and BAK, resulting in permeabilization of the outer mitochondrial membrane and execution of the intrinsic apoptotic process\textsuperscript{62}. Recently, IRF3, a transcription factor that induces IFN-β, has been shown to play a major role in cell death by way of its newly characterized BH3-only domain. IRF3 associated with the pro-apoptotic adaptor BAX and triggered apoptosis in murine embryonic fibroblasts and murine hepatocytes\textsuperscript{34,35}. However, it is not known whether other key signal transduction events in hepatocytes are required
for the pathogenesis of fibrosis and ALD. However, the liver is capable of
regeneration, leading to rapid repopulation of the organ through stem cell
differentiation into immature hepatocytes.
Figure 1.5 Apoptotic signaling via BH3-mediated association of IRF3 with BAX

Upon phosphorylation, IRF3 dimerizes and translocate to the nucleus, leading to type-I IFN induction. Under certain conditions, IRF3 co-localizes with BAX on the mitochondrial membrane through their BH3 motifs. This allows for activation of pro-apoptotic BAX, resulting in mitochondrial apoptosis through Caspase-8 and Caspase-3 cleavage.
The Inflammasome

Progression of ALD to an advanced stage is tightly linked to liver inflammation. Our lab previously demonstrated that the pro-inflammatory cytokine, interleukin (IL)-1β is required for the development of alcohol-induced liver inflammation, steatosis and injury and that the pathogenic effect of IL-1β is specific to resident liver macrophages. We also demonstrated that in the mouse model of ALD, the conversion of inactive pro-IL-1β to active IL-1β required the inflammasome, an intracellular multicomponent complex that triggers inflammation in response to endogenous danger signals. The inflammasome relies on pattern-recognition receptors (PPRs), such as TLRs and NOD-like receptors (NLRs) through the two-step process for active IL-1β synthesis. In the first step, microbial-derived signals upregulate pro-IL-1b mRNA, resulting in synthesis of inactive pro-IL-1β. This step is triggered by binding of bacterial LPS, derived from the gut, to the TLR4 on Kupffer cells, and provides an initial pro-inflammatory signal in ALD. Deficiency in TLR4 prevents upregulation of pro-IL-1b mRNA in the liver of alcohol-fed mice. In the second step, a variety of damage-associated molecular patterns (DAMPs) or ‘danger signals’ derived from host cells activate the inflammasome, resulting in cleavage of pro-IL-1β into the active IL-1β. The nature of endogenous DAMPs that activate inflammasome and IL-1β in ALD were not previously known.

We show that hepatocytes damaged by ethanol release the endogenous metabolic danger molecules, uric acid and ATP, which are recognized by liver
immune cells as inflammatory signals. Uric acid and ATP from damaged hepatocytes are required for processing and activation of IL-1β, a cytokine requiring inflammasome activation that is crucial in the pathogenesis of ALD. Our data suggests that the release of these molecules represent signals permissive for the pro-inflammatory effect of LPS in ALD.
Figure 1.6 Two-signal activation of inflammasome signaling

The NLRP3 inflammasome is a tightly-regulated inflammatory complex, activated through a two-signal system, relying on PAMPs and DAMPs. The first signal comes from exogenous gut-derived LPS, serving as a priming step. The second signal acts on NLRP3, can have many sources. In ALD, ATP and uric acid are endogenous DAMPs from damaged hepatocytes that activate NLRP3, resulting in cleavage and maturation of IL-1β.
Inflammation as a driver for ALD

Although hepatocyte damage from ethanol exposure is the first event observed in early ALD, inflammation through IL-1 and other cytokines plays a potentially bigger role in adding to its severity and complexity. The liver is an organ made up of 15-20% non-parenchymal cells, many of which are immune cells.

Increased levels of circulating LPS can be detected in humans and mice after chronic ethanol consumption, correlating with the severity of the disease. Interestingly, binge drinking also results in an increase in circulating LPS and other pathogen-associated molecular patterns (PAMPs). In addition to signaling through IRF3, TLR4-mediated signaling provokes a strong inflammatory response through MyD88-dependent and independent pathways. These branching signal transduction pathways result in activation of mitogen-associated kinases ERK and JNK, as well as in NFκB, TNFα and IL-6 release. Hepatocytes are particularly susceptible to TNF-mediated cell death through a priming effect from chronic ethanol exposure. Further adding to this potentiation of hepatocyte death, ethanol exposure has been shown to increase expression of TNFα-receptor. Lastly, monocyte chemoattractant protein 1 (MCP1) has been identified as an important cytokine in ALD, due to its dual role in recruiting more inflammatory cells to the liver as well as inducing steatosis.
Current treatments

A recent multi-center clinical trial (Cleveland Clinic, University of Louisville, UT Southwestern, and University of Massachusetts Medical School) aims to target inflammasome signaling in the treatment of severe alcoholic hepatitis. In addition to the standard of care, patients with severe alcoholic hepatitis will be administered a combination of Anakinra, an IL-1R antagonist\textsuperscript{64}, pentoxifylline, a phosphodiesterase inhibitor that suppresses TNF-α signaling\textsuperscript{85,86}, and zinc which attenuates gut barrier permeability\textsuperscript{87-89}. The goal of this multifaceted approach aims for a differential targeted approach at various components involved in the activation and escalation of IL-1 signal transduction, starting with gut-derived LPS, along with IL-1R and TNF-α -mediated amplification of inflammation.

Mouse models of ALD

Dynamic manipulation of organs and cells and components within as they respond to ethanol exposure, as well as accessing these samples for biochemical studies, is only possible by the use of \textit{in vivo} and \textit{in vitro} models. For \textit{in vivo} studies, there are various approaches used to study specific characteristics within the spectrum of alcoholic liver disease, ranging from acute exposure, to more complex events associated with chronic ethanol consumption, as well as acute-on-chronic ethanol injury, an amalgam of the latter two. We generally use female mice, due to their increased susceptibility to ethanol-
induced liver damage, in the range of six-to-eight weeks of age. Whenever possible, C57BL/6 mice are used as WT strain, although we always matched the background genotype of our knockout mice for every experiment. The C57BL/6 mice is the best for *ad libitum* feedings, since they offer the least resistance to ethanol compared to other strains.

### Acute Ethanol Binge

In order to study the early events associated with acute ethanol administration *in vivo*, an ethanol binge is administrated. We have used a standard dose given as a bolus at a dose of 5 g/kg using a gavage needle with ball tip. The control group receives a maltodextrin gavage at a dose of 9 g/kg. We found the ideal time-point for peak phosphorylation of IRF3 and cleavage of Caspase-1 is when mice are sacrificed 4 hours after binge. However, liver injury as measured by serum ALT peaks at 8 or 9 hours post binge. It is therefore important to tailor time-points for sacrifice to each specific readout as required by the experiment.

### Chronic Ethanol Administration

The standard method for chronic ethanol administration *ad libitum* to WT mice in the field of gastroenterology is using the Lieber-DeCarli method\textsuperscript{90,91}. This involved a ramp-up stage when mice are first exposed to control liquid diet on the first day along with chow. On the following day, chow diet is removed (0%
ethanol). The next day, Lieber-DeCarli diet with 1% ethanol is introduced, with an increase in one percent every subsequent day until reaching 5%. The first day with 5% ethanol is counted as the first full day of the ethanol feeding until reaching 4 weeks. There are variations of this feeding which can be extended for 6 weeks or shortened as needed. Regardless of the length of the feeding, the control group is given a Pair-fed diet where the calories are adjusted every day based on daily consumption changes in the ethanol group. The food is prepared fresh daily for both groups. The ethanol group has to have its calories supplemented with maltodextrin so that the ethanol and sugar always combine to a total of 355 kcal/liter. This adjustment is especially important during the ramp-up phase, when the amount of ethanol is changed daily but the number of calories remain the same.

**Acute-on-chronic Ethanol Administration**

Often with patients with alcoholic liver disease, the phenotype associated when present at the clinic is one of complexity associated with years of chronic ethanol consumption compounded with acute binging, resulting in a rapid inflammatory response and followed with deterioration of their condition. To best mimic this phenotype, the standard four week ethanol feeding has been adjusted to a ten day chronic ethanol feeding with a single binge at the end. In this model, mice are typically sacrificed 9 hours post binge, when peak liver injury is observed, but we tailored this to meet our needs depending on the experiment.
This model entails the use of 5% ethanol on day 1, without the incorporation of a ramp-up phase. The timing of this model results in the benefit of being a shortened period as well as increased liver injury and inflammation, highlighted by the presence of increased neutrophil infiltration to the liver – a cell-type not present in large numbers during a four-week ethanol feeding.

We also modified this model when trying to achieve a type of injury and inflammation greater than that observed with just ten days and a single binge. To best mimic acute AH, we administered a 4 week Lieber-DeCarli ethanol diet, and administered 3 ethanol binges during the final 3 days of the feeding\textsuperscript{93}. Moreover, as part of that study, we used this model of intense acute-on-chronic ethanol challenge to study the dynamics, over a period of seven days, of recovery from ethanol-diet withdrawal. During that time, we observed hepatocyte ballooning, liver infiltration by neutrophils days after the final ethanol dose, followed by impaired hepatocyte regeneration – characteristics previously unidentified in animal models of ALD yet important components of the disease in humans.
Figure 1.7 Mouse models of alcoholic liver disease

To model different characteristics of ALD in mice, we use different approaches for ethanol administration. The NIAAA acute-on-chronic model relies on 10 days of 5% ethanol *ad libitum* feeding. On the tenth day, an ethanol binge is administered, and mice are sacrificed typically 9 hours post binge (A). The traditional 4-week ethanol feeding *ad libitum* feeding incorporates a 5-day ramp-up period starting with 1% ethanol, escalating one-percent per day until reaching 5% ethanol, referred to as day 1 (B). A model of alcoholic hepatitis, this amalgam of the acute-on-chronic and traditional 4 week ethanol feedings, results in extensive inflammation and liver injury (C). These feedings rely on Lieber-DeCarli diet, with iso-caloric or ‘pair-fed’ controls.
A. Lieber-DeCarli chronic ethanol (5% vol/vol)
   Control liquid diet
   Acute ethanol gavage (5 g/kg)

   10 days
   Chronic Ethanol

   Day: 1 10
   Pair-fed

B. Lieber-DeCarli chronic ethanol (5% vol/vol)
   Control liquid diet

   Ramp-up
   Week 1  Week 2  Week 3  Week 4

   Day: 1 7 14 21 28
   Pair-fed

C. Lieber-DeCarli chronic ethanol (5% vol/vol)
   Control liquid diet
   Acute ethanol gavage (5 g/kg)

   Ramp-up
   Week 1  Week 2  Week 3  Week 4

   Day: 1 7 14 21 28
   Pair-fed
Other Mouse Models of Liver Injury

**Fibrosis**

Fibrosis represents a late-stage of liver disease that is common to all chronic liver diseases including viral hepatitis, alcoholic and non-alcoholic fatty liver disease, biliary liver diseases, and some genetic liver diseases. In spite of their specific etiologies, common denominators of fibrosis are shared among all these liver diseases, including liver inflammation and hepatocyte death.

Fibrosis results from chronic unresolved liver inflammation and may progress from fibrotic scarring to cirrhosis that ultimately leads to liver failure. Inflammation triggers liver fibrosis via a signaling event in which Kupffer cells activate hepatic stellate cells (HSC) to deposit collagen\(^{94,95}\). Liver dysfunction results from the fibrotic tissue distorting the liver parenchyma. This process involves a dynamic and complex series of multicellular events, involving inflammation and HSC activation. The hepatocyte is primarily responsible for metabolism and detoxification, and as such, it is often exposed to damage due to toxic metabolites and reactive oxygen species. However, the role of hepatocyte death in liver fibrosis has been only partially elucidated. Data available up to date demonstrate that hepatocyte apoptosis activates HSC via paracrine mechanisms. Alternatively, HSC can be directly activated by apoptotic bodies\(^{96}\).
Induction of fibrosis through carbon-tetrachloride

We used carbon-tetrachloride (CCl₄), a chemical that, in the short term, induces hepatocyte apoptosis⁹⁷, which is followed by secondary necrosis⁹⁸. CCl₄ is metabolized by CYP2E1, therefore the resulting phenotype bears many similarities with ethanol-induced liver injury. When administered repetitively, CCl₄ induces liver fibrosis⁹⁹. Using two models of CCl₄-induced liver injury, we investigated the complex multi-cellular events associated with fibrosis in a chronic model, as well as the tightly-controlled signal transduction events associated with early disruption of homeostasis and cell death in the acute model.

Acetaminophen-induced acute liver failure

Acetaminophen (APAP) overdose is one of the leading causes of acute liver failure, lead by a signaling cascade triggered by hepatocyte death and liver inflammation. Interestingly, APAP overdose results in hepatocellular damage that propagates even after the concentration of circulating xenobiotic has declined to unmeasurable levels¹⁰⁰. This suggests a potential role for propagation of preexisting injury, rather than simply de novo direct injury.

Much like ethanol-induced liver injury, APAP pathogenesis exhibits spatial heterogeneity, due in part to varying expression levels of CYP2E1¹⁰¹-¹⁰⁴. This zonation of APAP-induced injury occurs during the initial phase of injury¹⁰⁵,
resembling an exaggerated phenotype observed with ethanol and CCl₄ administration. The second phase of injury is poorly defined, however. In this model, mice are starved 15 hours (overnight) before a single intraperitoneal APAP administration. The removal of food allows for glutathione depletion, exacerbating liver injury. A dose of 500 mg/kg is a commonly used lethal dose over the course of 24 hours.
CHAPTER TWO: IRF3 SIGNALING IN ALD

IRF3 is required for the development of ALD in mice \(^{26}\), but the mechanisms underlying its pathogenic role in the liver are not known. To gain a better insight into its pathology, we investigated mechanisms upstream and downstream of IRF3 phosphorylation in various models of ALD, initially focusing on the early events after ethanol exposure as a simplified model.

Results

Pathogenic role of IRF3 in ALD

We first tested the phenotype of whole-body IRF3-knockout (KO) mice after a 4 week Lieber-DeCarli chronic ethanol feeding. IRF3-KO mice showed full protection from ethanol-induced liver damage, compared to WT ethanol-fed mice (Fig. 2.1A). Consistent with its role in induction of TNF-\(\alpha\) \(^{107}\), an absence of IRF3 signaling led to a protection from liver inflammation compared to WT mice, as seen by no increase in expression of pro-inflammatory genes \(Tnfa\), \(pro-IL1b\) and \(Mcp1\) mRNA (Fig. 2.1B). However, IRF3-KO mice had a similar increase to WT mice in serum endotoxin (Fig. 2.1C). We chose the 4-week Lieber DeCarli model because as the standard chronic ALD model, it would reveal if IRF3 played any role in its pathogenesis. Together, this data suggested that ethanol-induced liver injury and inflammation in this model was driven by IRF3 signaling, but not determined entirely by levels of circulating lipopolysaccharide.
**Figure 2.1. Profile of chronic ethanol injury in WT and IRF3-KO mice**

A 4 week Lieber-DeCarli ethanol diet was administered to WT and IRF3-KO mice. Blood and liver tissue were isolated. Liver injury was evaluated using serum ALT (A); Liver inflammation was measured from expression of *Tnfa*, *pro-IL1β*, and *Mcp1* mRNA using RT-PCR (B); Bacterial lipopolysaccharide (endotoxin) was measured from serum (C). *n = 6-10 mice per group. * P < 0.05 vs baseline.*
The 4-week model of ALD represents a compound outcome of both acute and chronic events. We sought to understand the dynamics of liver injury and phosphorylation of IRF3 after initial ethanol exposure. For this, we used the ethanol binge model, where WT mice were given an oral bolus of 5 g/kg ethanol or iso-caloric maltodextrin. Mice were sacrificed at various time-points, as indicated. We detected a rapid increase in serum endotoxin, as early as 1 hour, peaking at 2 hours, and normalizing 8 hours after the ethanol binge (Fig. 2.2A). Liver injury, as measured by serum ALT, followed a gradual increase peaking at 8 hours after the binge, which then normalized by hour 16 of the experiment (Fig. 2.2A). During this time-course experiment, we detected no increase in liver expression of pro-inflammatory Tnfa mRNA (Fig. 2.2A), which supported our findings from chronic feeding (Fig. 1.2B). We did, however, detect an early increase in Ifnb liver mRNA, as early as 2 hours, peaking 4 hours after ethanol administration. INF-β, a type-I IFN, is induced upon IRF3 phosphorylation and translocation to the nucleus. This early increase in Ifnb mRNA suggested early involvement of IRF3 in a single-binge ethanol-induced liver injury model.
Figure 2.2. Profile of acute ethanol injury and inflammation in WT mice

A single dose of EtOH (5 g/kg) was administered to WT. Blood and liver tissue were isolated at indicated time points. Liver injury was evaluated using serum ALT (A, right in red); Bacterial lipopolysaccharide (endotoxin) was measured from serum (A, left in blue). Liver inflammation was measured from expression of Ifnb and Tnfa was measured from liver lysate using RT-PCR (B). n = 5-8 mice per group. * P < 0.05 vs baseline.
Early ALD is a hepatocyte-driven phenotype

We directly tested whether Kupffer cells were responsible for this early damage from ethanol administration. We depleted resident macrophages from the liver via clodronate injection, which we confirmed two days later via F4/80 immunohistochemistry (Fig. 2.3A). Depletion of Kupffer cells by clodronate did not affect liver injury assessed by ALT levels (Fig. 2.3B). As noted earlier, acute liver injury induced by a single dose of ethanol was not accompanied by inflammation (Fig. 2.1B). Here, we found that initial liver injury developed independent of Kupffer cells, a major source of inflammatory cytokines in the liver. We then tested if IRF3 was needed for liver injury in a single ethanol binge, to help isolate the early events from ethanol exposure from the more intricate associated with the chronic disease that involve recruitment of more immune cells to the liver. We compared WT mice and IRF3-KO mice to mice deficient in inflammatory signaling. The extent of protection from liver damage in IRF3-KO mice was superior to that observed in other strains deficient in innate immune signaling. For example, absence of inflammasome components, Caspase-1 (Casp-1) which is required for IL-1β maturation, or inhibition if IL-1R with IL-1R antagonist (IL-1Ra, also known as Anakinra) \(^{64}\), surprisingly did not protect from liver injury (Fig. 2.3C). This data suggested that acute alcoholic liver injury is dependent on IRF3, but independent of Kupffer cells and inflammation.
**Figure 2.3 Acute liver injury develops independently of liver immune cells**

WT mice received i.v. clodronate or PBS (control). Two days later, depletion of immune cells was verified by histology of liver sections through F4/80 immunostaining (A). A single dose of EtOH (5 g/kg) was administered to WT mice previously treated with clodronate or PBS. Blood was isolated at indicated times and liver injury was evaluated by serum ALT (B). A single dose of EtOH (5 g/kg) was administered to WT, Caspase-1 (Casp1)-KO, IRF3-KO, and mice pre-treated with IL-1Ra (Anakinra). Blood was isolated at indicated times and liver injury was evaluated by serum ALT. (C) n = 5 mice per group. * P < 0.05 vs baseline.
Depletion of Kupffer cells

Control  |  Clodronate

F4/80

Serum ALT

PBS  |  Clodronate

0  |  50  |  100  |  150  |  200

0  |  2  |  4  |  8  |  16

A

C

WT  |  Casp1-KO  |  IL-1Ra treatment  |  IRF3-KO

IRF3-KO vs. WT: global $P < 10^{-5}$
Phosphorylation dynamics

We had established a critical pathogenic role for IRF3 in the liver upon ethanol-induced injury. We previously identified that a single binge had resulted in an *Infb* mRNA increase, suggestive of IRF3 activation. However, we did not know if involvement of IRF3 in ALD involved its phosphorylation and activation. We tested dynamics of IRF3 phosphorylation in total liver in WT mice – and for that, we needed to perform ethanol exposure to a single binge over various time points. We detected rapid phosphorylation in liver after an ethanol binge at 1 hour. This signal remained elevated up to 8 hours, after which it decreased back to baseline (Fig. 2.4A-B). Given the early induction of *Infb* mRNA, as well as subsequent liver injury within the first hour, we hypothesized that IRF3 phosphorylation would occur in hepatocytes. To test this, we administered an ethanol gavage to WT mice, isolated hepatocytes through liver perfusion 2 hours later. We detected an increase in phosphorylated IRF3 in these hepatocytes (Fig. 2.4C-D). We found a similar dynamic of IFN-β production as that previously observed with *Infb* mRNA (Fig. 2.4E).

This was the first reported case of IRF3 phosphorylation in hepatocytes, a previously well-studied player of innate immunity in immune cells. We confirmed IRF3 protein expression in primary murine hepatocytes (Fig. 2.4F). I will use this opportunity to show relative levels of other important signaling components in the IRF3 signaling axis, cGAS and STING, which are all expressed by WT C57BL/6 mice.
It is important to highlight a recent publication that stated there was no detectable STING protein expression in WT primary murine hepatocytes\textsuperscript{108}. It should be noted, however, that this publication does show increased STING protein expression in murine liver lysate compared to STING\textsuperscript{Gt/Gt} mice, a genotype with deficient STING signaling first identified in a mutational screen\textsuperscript{109}. This report did detect expression of cGAS and IRF3 in primary murine hepatocytes.
Figure 2.4 IRF3 is activated in hepatocytes after acute ethanol in vivo

One dose of EtOH (5 g/kg) was administered in vivo to WT mice. Livers were isolated at indicated time points and whole-cell lysate was probed for phosphorylated IRF3 (p-IRF3) and total IRF3 via western blot (A). Densitometry was calculated using ImageJ (B). Two hours after a binge, hepatocytes were isolated from the liver. Phosphorylated IRF3 and total IRF3 were analyzed by western blot (C). n = 3 mice per group. Densitometry was calculated using ImageJ. (D). Serum was isolated and serum IFN-β was measured by ELISA (E). n = 5-8 mice per group. Hepatocytes were isolated from livers of WT, cGAS-KO, STING-KO, and IRF3-KO six to ten week-old female mice, all on C57BL/6 background. Protein expression of cGAS, STING and IRF3 in hepatocytes was assessed via western blot (F). * P < 0.05 vs baseline
Primary murine hepatocytes

**Immunoblotting**

A

- **A**: Immunoblotting

- **B**: densitometry

- **C**: Primary murine hepatocytes

- **D**: Densitometry: p-IRF3/IRF3

- **E**: serum IFN-β

- **F**: Primary murine hepatocytes
Pathogenic effect of IRF3 independent of TRAM and IFNAR

We asked whether the pathogenic role of IRF3 in ALD was mediated by its upstream activator TRAM, the MyD88-independent adaptor which bridges TLR4 with downstream signaling that induces phosphorylation of IRF3 in immune cells. Interestingly, no protection from alcohol-induced liver inflammation or alcohol-induced acute or chronic liver injury or inflammation was observed in mice deficient in TRAM (Fig. 2.5A-C). We also found no protection in these mice from acute ethanol-induced liver injury compared to the WT group (Fig. 2.5D). We found a similar lack of protection in mice deficient in either TLR4 or TRIF, another MyD88-independent adaptor. Since pathogenicity seemed driven by IRF3 and not upstream activators, we tested downstream signaling of IRF3. We found that deficiency of the type-I IFN receptor (IFNAR1) failed to provide protection from acute or chronic alcohol-induced liver injury and inflammation (Fig. 2.5E and 110). There was no protection from ALD in mice deficient in IRF7, a type-I IFN-inducing transcription factor distinct from IRF3. Collectively, these findings demonstrated that IRF3 determines pathology in the early stage of ALD, independently of inflammation or type-I IFN signaling. Our data also suggested that the signal for activation of IRF3-dependent ALD in the early phase of alcohol-induced liver injury was not mediated by TLR4 or its adaptor TRAM.
**Figure 2.5 Liver injury is independent of TRAM and IFNAR signaling in ALD**

A 4 week Lieber-DeCarli ethanol diet was administered to WT and TRAM-KO mice. Blood and liver tissue were isolated. Liver injury was evaluated using liver histology with H&E staining (A), and serum ALT (B); Liver inflammation was measured from expression of *Tnfa* mRNA using RT-PCR (C); n = 6-10 mice per group. A single dose of EtOH (5 g/kg) was administered to WT or TRAM-KO mice. Blood was isolated at indicated times and liver injury was evaluated by serum ALT (D). A single dose of EtOH (5 g/kg) was administered to WT or IFNAR1-KO mice. Blood was isolated at indicated times and liver injury was evaluated by serum ALT (E). * P < 0.05 vs baseline. # P < 0.05 vs TRAM-KO baseline.
1x intragastric dose of ethanol
Association with mitochondria

In trying to gain a better understanding of the pathogenic role of IRF3, we focused on the recently described pro-apoptotic role of IRF3\textsuperscript{34,35}. We hypothesized that IRF3 partners with BAX to translocate to the mitochondria and initiate apoptosis of hepatocytes.

Phosphorylation of IRF3 in the liver occurred early after a single intragastric dose of alcohol (Fig. 2.4A), which preceded the development of significant liver injury. This was accompanied by cleavage of caspases 8 and 3, suggestive of apoptosis in the liver (Fig. 2.6A,B). Notably, we found that following ethanol administration, IRF3 associated with the apoptosis-initiating caspase 8 and with the mitochondria-permeabilizing protein BAX in the liver (Fig. 2.6C,D). Together, these data demonstrated that early after administration of ethanol, IRF3 is activated and interacts with the apoptotic signaling pathway in the liver, leading us to hypothesize that IRF3 regulates apoptosis in hepatocytes.
Figure 2.6 IRF3 induces apoptotic signaling through association with BAX in early ALD

One dose of EtOH (5 g/kg) was administered *in vivo* to WT mice. Livers were isolated at indicated time points and whole-cell lysate was probed for cleaved Caspase-8 and cleaved Caspase-3 via western blot (A). Densitometry was calculated using ImageJ (B). Immunoprecipitation performed of IRF3 in whole-cell liver lysate at indicated time-points, and probed for associated with Caspase-8 p43 fragment and BAX (C). Densitometry was calculated using ImageJ (D). n = 3 mice per group. * P < 0.05 vs baseline
A

**Immunoblotting**

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B

**densitometry**

- Fold-increase (normalized to β-tubulin)
- cleaved Casp-8 p43
- cleaved Casp-3 p17/19

C

**Immunoprecipitation**

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D

**densitometry**

- Fold-increase (normalized to total IRF3)
- IRF3 + Casp-8 p43
- IRF3 + Bax
We further investigated whether IRF3 was required for hepatocyte apoptosis using the Fas-activating Jo2 antibody. Hepatocytes have a pronounced susceptibility to Fas-mediated apoptosis. We found that administration of Fas-activating Jo2 in WT mice resulted in early phosphorylation of IRF3 in the liver followed by apoptosis (Fig. 2.7A,B) and liver injury (Fig. 2.7C). When Jo2 was injected in IRF3-KO and WT mice, IRF3-KO mice were completely protected from liver injury and death compared to WT mice (Fig. 2.7C,D). Specifically, compared to IRF3-KO mice, WT mice had elevated serum ALT (Fig. 2.7C), decreased survival (Fig. 2.7D), and increased pro-apoptotic signaling in the liver, as measured by cleavage of Caspase-8, -3, and Poly(ADP-ribose) polymerase (PARP) (Fig. 2.7E). Active Caspase-3 and Caspase-7 lead to cleavage of PARP, an enzyme with nuclear activity in response to DNA strand breaks.\textsuperscript{111,112}

Collectively, this data demonstrated that IRF3 is expressed in hepatocytes and is required for hepatocyte apoptosis in response to ethanol. Our results demonstrate that the pathogenic effect of IRF3 in acute and chronic ALD is mediated by intrinsic IRF3-dependent hepatocyte apoptosis and is independent of the role of IRF3 in inflammatory or type-I IFN responses.
Figure 2.7 IRF3 is required for apoptosis in the liver

One dose of Jo2 antibody 0.5 mg/kg, i.p. was administered to WT mice. Livers were isolated at indicated time points and whole-cell lysate was probed for phosphorylated IRF3 (p-IRF3), total IRF3 and cleaved PARP via western blot (A). Densitometry was calculated using ImageJ (B). n=3 per time-point. * and # P < 0.05 vs baseline. One injection of Jo2 antibody (FasL) was administered to WT and IRF3-KO mice. Liver injury was assessed via serum ALT at 8 hours (C). Some mice were evaluated for survival over 48 hours (D). n= 24 (WT), n=13 (IRF3-KO). Other mice were sacrificed 8 hours post injection. Whole-cell liver lysates were evaluated for cleaved PARP, cleaved Caspase-3, and cleaved Caspase-8 via western blot (E). n = 3-5 mice per group.
A

**Immunoblotting**

- **p-IRF3**
- **total IRF3**
- **clv PARP**
- **β-tubulin**

**Time (hrs):** 0, 0.5, 1, 2, 4

B

**Densitometry**

- **pIRF3/total IRF3 fold-increase**
- **cleaved PARP/β-tubulin fold-increase**

**Time (hrs):** 0, 0.5, 1, 2, 4

C

**ALT**

- **Vehicle**
- **FasL**

**ALT (U/L):**

- WT: 25-40
- IRF3-KO: 0-5

D

**Mouse survival**

- **WT**
- **IRF3-KO**

**% survival:**

- P=0.003

E

**Western Blot Analysis**

- **Wild-type**
  - saline
  - FasL
- **IRF3-KO**
  - saline
  - FasL

**Proteins:**

- cleaved PARP
- Casp-3 full-length
- cleaved p18
- Casp-8 cleaved p43
- β-Actin
STING activation

Next, we sought to identify the mechanism of IRF3 activation in ALD. Based on our previous observations, we hypothesized that a TRAM-independent mechanism was responsible for IRF3 activation in ALD. Although administration of Jo2 induced phosphorylation of IRF3 in the liver, we rejected the hypothesis that Fas signaling would be a major source of early IRF3 activation in ALD because significant upregulation of FasL in the liver occurred late after ethanol administration.

Analysis of the subcellular baseline distribution of IRF3 in the livers of control WT mice revealed that IRF3 was present not only in the cytoplasmic fraction, but also in mitochondrial and ER extracts (Fig. 2.8A). To study the functional significance of this finding, we analyzed the subcellular localization of IRF3 after a single dose of ethanol, and found that phosphorylation of IRF3 was detected in the whole-cell liver extract (Fig. 2.8B), nucleus (Fig. 2.8C), ER (Fig. 2.8D) and mitochondria (Fig. 2.8E). The western blot for total IRF3 in the ER and mitochondrial extracts showed a double band, which was due to the presence of phosphor-IRF3, which was first probed for on the same membrane. In instances like these, phosphor-IRF3 is the top band, while total IRF3 is below. The presence of phospho-IRF3 in the nucleus was consistent with the transcriptional role of activated IRF3\textsuperscript{107} and the early alcohol-induced Ifnb (Fig. 2.2B). Interestingly, the strength of the Tata-binding protein (TBP) in the nuclear extract decreased at 8 hours (Fig. 2.2C). The presence of phospho-IRF3 in the
mitochondria further supported the involvement of IRF3 in the mitochondrial pathway of hepatocyte apoptosis (Figs. 2.6 and 2.7).

The finding of phospho-IRF3 in the liver ER (Fig. 2.8D) was unexpected and prompted us to search for potential implications of this phenomenon. It has been reported that ER serves as a platform for activation of IRF3 by cytosolic RNA or DNA of viral origin\textsuperscript{34,35}, and that during certain viral infections, IRF3 associates with ER via binding to Stimulator of IFN gene (STING, also known as TMEM173 or MPYS), an adaptor molecule residing in the ER membrane\textsuperscript{29}. To explore whether interaction between IRF3 and STING occurred in the liver, we performed immunoprecipitation and found that following administration of ethanol, IRF3 associated with STING in the whole-cell liver lysate (Fig. 2.8F) and in the ER extract (Fig. 2.8D). IRF3 also bound to phospho-TBK1 (Fig. 2.8F,H), which is a kinase required for IRF3 phosphorylation\textsuperscript{29}. Notably, the association of IRF3 with STING or p-TBK1 in the ER occurred rapidly after alcohol administration, at the same time point as the binding of IRF3 to BAX (Fig. 2.6C). Interestingly, although STING is also present in the outer mitochondrial membrane\textsuperscript{113}, we did not see any association between IRF3 and STING or TBK1 in the liver mitochondrial fraction (Fig. 2.8G).
Figure 2.8 IRF3 associates with STING in the hepatic ER after acute ethanol

Unstimulated livers from WT mice were isolated and were probed for IRF3 in whole-cell, cytoplasmic, mitochondrial, ER, and nuclear extracts via western blot. β-tubulin, porin, GRP78, and TBP were used as loading controls for cytoplasmic, mitochondrial, ER, and nuclear fractions, respectively (A). WT mice received a single dose of ethanol. At indicated time-points, presence of phosphorylated IRF3 (p-IRF3) protein was assessed in whole-cell, nuclear, ER and mitochondrial extracts (B-E). WT mice received a single dose of ethanol. At indicated time-points, IRF3 from whole-cell, mitochondrial, and ER fractions was immunoprecipitated and probed for associated with phospho-TBK1 and STING (F-H). n=3 mice per time-point. * P < 0.05 vs baseline.
The presence of IRF3 in the ER upon ethanol administration, and its connection with STING suggested a galvanizing link through this association. To test this, we used Tmem173<sup>gt</sup> mice deficient in STING signaling<sup>109</sup>. We found that Tmem173<sup>gt</sup> mice were susceptible to chronic ethanol administration by day 28 (4 weeks), but had decreased levels of liver injury at day 14 compared to their WT controls (Fig. 2.9A). We tested if this early protection could be replicated with the acute ethanol binge model. Indeed, Tmem173<sup>gt</sup> mice had decreased liver injury compared to WT mice (Fig. 2.9B). We tested if this slight protection in liver injury in Tmem173<sup>gt</sup> mice was mediated by IRF3. We found Tmem173<sup>gt</sup> mice had no detectable levels of ethanol-induced IRF3 phosphorylation and Caspase-3 cleavage after 2 weeks of Lieber-DeCarli ethanol feeding (Fig. 2.9C,D).

We hypothesize that the liver injury observed in the Tmem173<sup>gt</sup> mice at 4 weeks, equal to their WT counterparts, may be due to activation of TLR4 signaling. These overlapping pathways involved in IRF3 activation may be able to compensate over the course of a chronic injury. The involvement of TLR4 in ALD has been well documented by others<sup>24</sup>, in a 4 week model which was described by the study’s authors as early ALD. Here, we describe early ALD as earlier events. Additionally, it is possible that the lack of protection from late alcohol liver damage in Tmem173<sup>gt</sup> mice was attributable to the upregulation of inflammatory cytokines, which have not been reported to be controlled by STING-dependent signaling. Finally, these findings demonstrated that early after ethanol...
administration, IRF3 interacts with the adaptor STING in the ER, and raised the question of whether ER stress was a trigger for IRF3 phosphorylation in ALD.
Figure 2.9 STING activates pro-apoptotic IRF3 in early ALD

WT and Tmem173\textsuperscript{gt} mice were fed an ethanol Lieber-DeCarli diet for 4 weeks. Liver injury was assessed by serum ALT at day 14 and day 28 (A). WT and Tmem173\textsuperscript{gt} mice received a single dose of ethanol. At indicated time-points, liver injury was assessed by serum ALT (B). WT and Tmem173\textsuperscript{gt} mice were fed an ethanol Lieber-DeCarli diet for 2 weeks. Presence of phosphorylated IRF3 (p-IRF3) (C) and cleaved Caspase-3 protein was assessed in whole-cell liver lysate (C,D). n=5-9 mice per time-point. * P < 0.05 vs baseline.
A  4 wks chronic ethanol

serum ALT

B  1x ethanol binge

serum ALT

global $P = 2.3 \times 10^{-5}$

C  2 wks chronic ethanol

2 wks chronic ethanol

D  2 wks chronic ethanol

2 wks chronic ethanol

C

D

2 wks chronic ethanol

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Activation of IRF3 via ER stress

A broad variety of stressors perturb ER function in ALD, including oxygen deprivation, dysregulation of calcium signaling and accumulation of misfolded proteins\textsuperscript{114}. Disruption of intracellular homeostasis can lead to an accumulation of misfolded proteins associated with the unfolded protein response (UPR) in the ER, also known as ER stress. Excessive ER stress is associated with pro-apoptotic signaling and can be pathogenic\textsuperscript{115,116}. These events initiate homeostatic responses but also contribute to liver pathology\textsuperscript{117}.

ER stress triggers STING activation

We used upregulation of \textit{Xbp1} mRNA splicing (s\textit{Xbp1}), a marker of early ER stress \textsuperscript{118,119}. We found that administration of a single dose of ethanol induced early ER stress in the liver, as demonstrated by increased splicing of \textit{Xbp1} mRNA (s\textit{Xbp1}) (Fig. 2.10A), a component of the ER stress-associated response\textsuperscript{120}. Analysis of cell populations in the liver demonstrated that increased splicing of \textit{Xbp1} occurred in hepatocytes, but not in LMNCs of mice exposed to acute alcohol (Fig. 2.10B), which was consistent with the dominant role of hepatocytes in the metabolism of ethanol. In fact, when primary hepatocytes were stimulated with ethanol, the observed ER stress occurred independently of acetaldehyde. This finding was consistent with the findings from others that the
pathogenic effects from ethanol are mediated by CYP2E1 independently of acetaldehyde, which are themselves enhanced by LPS and TNFα\(^{121,122}\).

We found a similar, yet stronger phenotype in CCl\(_4\)-induced acute hepatocyte injury. Intraperitoneal CCl\(_4\), which is metabolized in a similar CYP2E1-dependent manner as ethanol, induces an early ER stress response. In this model of liver injury, when analyzed livers of WT mice at various time points after a single administration of CCl\(_4\), we found an early increase in \(sXbp1\) mRNA splicing one and two hours after administration of CCl\(_4\) (**Fig. 2.10C**). Analysis of cell populations from murine livers showed that CCl\(_4\) selectively upregulated \(Xbp1\) mRNA splicing in hepatocytes but not in LMNCs (**Fig. 2.10D**)

Previous reports have shown that ER stress results in IRF3 activation in mouse embryonic fibroblast, and that \(Xbp1\) splicing enhances the IFN-β response in immune cells\(^{123,124}\). To test if direct induction of ER stress can lead to IRF3 phosphorylation, we treated WT primary hepatocytes with thapsigargin *in vitro* and found an early increase in phosphorylated IRF3 (**Fig. 2.10E**). Inhibition of Tank-binding kinase 1 (TBK1), a kinase responsible for IRF3 phosphorylation, with BX795 showed a dose-dependent reduction in IRF3 phosphorylation in thapsigargin-treated hepatocytes (**Fig. 2.10E**). This finding indicated that activation of IRF3 in hepatocytes by ER stress is dependent on STING and TBK1. Next, we observed that thapsigargin failed to induce IRF3 phosphorylation in hepatocytes isolated from the Tmem173\(^{ol}\) mice (**Fig. 2.10F**), which lack the STING protein.
Figure 2.10 ER stress activates IRF3 via TBK1 and STING in early ALD

WT mice received a single dose of ethanol. At indicated time-points, presence of ER stress was measured by evaluation of spliced Xbp1 (sXbp1) mRNA in liver lysate (A) or isolated hepatocytes and liver mononuclear cells (LMNCs) (B) via PCR on agarose gel. WT mice received a single dose of i.p. injection of CCl4. At indicated time-points, presence of ER stress was measured by evaluation of spliced Xbp1 (sXbp1) mRNA in liver lysate (C) or isolated hepatocytes and liver mononuclear cells (LMNCs) (D) via PCR on agarose gel. N=3 mice per time-point. WT primary hepatocytes were pre-treated with DMSO or BX795 (10 μM and 100 μM), and stimulated with thapsigargin (1μM). Whole-cell lysate was analyzed for p-IRF3 by western blot (E). Primary hepatocytes from WT and Tmem173gt mice were stimulated with thapsigargin (1μM) and analyzed for p-IRF3 and for GRP78 (F). * P < 0.05 vs baseline.
**A** Liver sXBP1

1x intragastric dose of ethanol

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**B** Cell-specific sXBP1

1x intragastric dose of ethanol

Hepatocytes control vs. EtOH

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**C** Liver sXBP1

1x i.p. dose of CCl4

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**D** 1x i.p. dose of CCl4

Hepatocytes vs. LMNCs

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**E** WT Primary Hepatocytes + Thapsigargin

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**F** WT or Tmem173R mouse hepatocytes

Thapsigargin

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ER stress as a therapeutic target in ALD

To investigate the role of endoplasmic reticulum (ER) stress on liver inflammation and hepatocyte death, we will administer animals with ER stress inhibitors (ERSI) orally before and/or during acute/chronic ethanol administration. Alcohol consumption causes ER stress in the liver. ER stress is accompanied by the unfolded protein response, which serves to maintain homeostasis in the cell to aid in recovery\textsuperscript{119}. High levels or sustained ER stress is associated with cell death\textsuperscript{115,125,126}. More recently, ER stress has been directly linked with activation of STING in the ER, resulting in phosphorylation of IRF3\textsuperscript{33,124}, a critical innate immune transcription factor that can trigger apoptosis in hepatocytes through association with pro-apoptotic BAX\textsuperscript{110,127}. We hypothesize that pharmacological inhibition of ER stress via administration of a small molecule protein-folding chaperons, such as 4-phenylbutyrate (4PBA), will protect cell from STING and IRF3-mediated apoptotic signaling. The use of this and other inhibitors, like tauroursodeoxycholic acid (TUDCA)\textsuperscript{128} has been shown to be beneficial in a model of Type 2 diabetes\textsuperscript{129} and in a model of NASH\textsuperscript{130}. 4PBA has been shown to be effective by administration via gavage or in the drinking water, as well as through intraperitoneal injection. Here, we tested in a pilot experiment if oral administration of the drug was equally as effective as self-administration by the mice. For this, we mixed 4PBA directly in the liquid diet and found comparable decrease in spliced Xbp1 protein in the liver as mice that received a daily gavage of 4PBA (data not shown). For subsequent experiments,
WT mice were fed Lieber-DeCarli liquid diet (PF or ethanol) containing 4PBA at a
dose of 2 mg/kg daily as part of an acute-on-chronic ethanol diet. This model of
ethanol-induced liver injury was chosen because we had established that IRF3
was involved in early late chronic liver injury from ethanol, whereas STING
played a major role after acute-only or acute-on-chronic liver injury, but not at the
culmination of a four-week ethanol feeding. Therefore, within this model, we
could carefully control for time-sensitive dynamics of IRF3 phosphorylation,
which peaked at 4hrs, while still testing for both, acute and chronic events,
through the administration of a single binge as part of or independently from a
ten-day ethanol administration model. We found that administration of 4PBA as a
preventative measure, effectively reduced ethanol-induced liver injury (Fig.
2.11A). Importantly, 4PBA was effective at reducing phosphorylation of IRF3 4
and 9 hours post ethanol binge in liver lysate (Fig. 2.11B). As a positive control,
we found that daily dose of 4PBA at 2 mg/kg was effective in reducing
tunicamycin-induced ER stress in the liver (data not shown). Interestingly, we
found elevated levels of phosphorylated IRF3 in liver lysate 12 hours after
tunicamycin intraperitoneal injection (Fig. 2.11B) when injected at 2 mg/kg131.
Figure 2.11 Therapeutic inhibition of ER stress attenuates liver injury and IRF3 phosphorylation

WT mice were fed a 10-day acute-on-chronic Lieber-DeCarli ethanol diet containing 4PBA (2 mg/kg/day). Mice were sacrificed 4 and 9 hours after an ethanol binge. Liver injury was assessed by serum ALT (A). Phosphorylation of IRF3 from whole-cell liver lysate of mice treated with or without 4-PBA (B). Mice injected with tunicamycin (TM) were used as positive control of ER stress.
A

![Bar graph showing Serum ALT (U/L) with 4-PBA treatment compared to EtOH binge.](image)

B

![Western Blot showing p-IRF3 and IRF3 levels with different treatment conditions.](image)
Building upon previous findings, our data indicate that acute administration of ethanol or CCl₄ results in hepatocyte-specific ER stress and phosphorylation of IRF3 \textit{in vivo}, and that \textit{in vitro} inhibition of a kinase upstream of IRF3 inhibited phosphorylation of IRF3 induced by ER stress. Our data support the hypothesis that liver injury activates STING and IRF3 in hepatocytes via ER stress, as summarized in Figure 2.12.

Collectively, our data demonstrated that activation of IRF3 in ALD is triggered by the ER stress in hepatocytes via the adaptor STING. As such, ER stress is a potential therapeutic target in ALD. Thus, STING and IRF3 are key determinants of ALD, linking ER stress signaling with the mitochondrial pathway of hepatocyte apoptosis.
Hepatocellular injury, from ethanol or CCl₄, induces ER stress and STING activation in the ER. This leads to recruitment of TBK1, which phosphorylates and activates IRF3. This signaling sequence results in IRF3 associating with BAX on the mitochondrial membrane, and an activation of apoptosis through Caspase-8 and Caspase-3.
hepatocellular injury

Endoplasmic Reticulum

ER stress

STING

Xbp1 splicing

Cytoplasm

TBK1

IRF3

IRF3

Type-I IFN

Mitochondrion

BH3

RF3

BAX

active Caspase-3

Hepatocyte Apoptosis

Secondary Necrosis

Liver Fibrosis
The role of cGAS

There are several intracellular double-stranded (ds) DNA sensors that activate STING signaling in response to viral infection. The cyclic dinucleotide 2’3’-cGAMP, synthesized by the dsDNA cytosolic sensor cGAMP synthase (cGAS), activates STING. We hypothesized that cGAS may be an upstream activator of STING and IRF3 in ALD. We found complete protection from liver injury and inflammation in IRF3-KO mice. However, only partial attenuation of liver injury was observed in STING-deficient mice. The strongest protection was observed early during the course of a chronic ethanol feeding or from acute ethanol binge. For this reason, we decided to test the role cGAS using the ten-day NIAAA model of acute-on-chronic ethanol injury with slight modifications (Fig. 1.7A). We would incorporate a group of mice that did not receive a binge after 10 days of Lieber-DeCarli ethanol diet, allowing us to test the effects of acute injury separately from the chronic. In addition, some mice were sacrificed 4 hours post ethanol binge, rather than the 9 hours, as is recommended by the NIAAA model. These two time-points would allow us to observe peak IRF3 phosphorylation, which occurs in the first few hours post binge, while the 9-hour time point would allow us to observe overall effects associated with peak liver injury. We found that cGAS-KO mice had decreased levels of serum ALT, improved liver morphology and decreased F4/80 mRNA after acute binge at 4 and 9 hours (Fig. 2.13A-C). Interestingly, cGAS-KO mice that received only
chronic Lieber-DeCarli ethanol diet for 10 days (without a binge) had comparable liver injury as WT mice.
Figure 2.13 cGAS deficiency protects from acute-on-chronic liver injury

WT and cGAS-KO mice were fed an ethanol Lieber-DeCarli diet for 10 days with a single ethanol binge on day 10. Some mice were sacrificed 4 or 9 hours post ethanol binge. Pair-fed mice received a binge of iso-caloric maltodextrin. Liver injury was assessed by serum ALT (A) and H&E liver histology (200X magnification) (B). Livers were analyzed for F4/80 mRNA expression (C). n=5-7 mice per time-point. * P < 0.05 vs baseline.
A

10 days EtOH

serum ALT (U/L)

PF 0hr 4hr 9hr

WT cGAS-KO

* NS **

+ 1x EtOH binge

B

PF No Binge 4hr Binge 9hr Binge

WT cGAS-KO

C

F4/80

fold-increase [mRNA]

PF No Binge 4hr Binge PF No Binge 4hr Binge

10-day Ethanol 10-day Ethanol
Given that cGAS-KO mice were not protected from 10 days of chronic ethanol, but had strong protection from the acute effects of acute-on-chronic ethanol, we then tested if cGAS-KO were protected from liver injury in the acute ethanol binge model of early ALD. We found lower levels of serum ALT after an ethanol binge in cGAS-KO mice compared to their WT controls (Fig. 2.14A). We then tested if cGAS signaling was a determinant for ethanol-induced IRF3 phosphorylation in the liver. We found no detectable increase in phosphorylated IRF3 in liver from cGAS mice compared to WT mice (Fig. 2.14B). Lastly, we tested if cGAS mediated the apoptotic role associated with IRF3 activation. We found no increase in Caspase-3 cleavage in livers from cGAS-KO mice after a single ethanol binge compared to WT mice. Together, this data suggested that IRF3 phosphorylation and concomitant pro-apoptotic signaling is mediated by cGAS in early ALD. This phenotype was present from a single stand-alone binge or as part of an acute-on-chronic ethanol injury.
Figure 2.14 cGAS mediates acute ethanol-induced IRF3 phosphorylation

WT and cGAS-KO mice were given a single ethanol dose. Liver injury was assessed by serum ALT (A). Livers were analyzed at indicated time-points and tested for phosphorylated IRF3 (p-IRF3) (B) and cleaved Caspase-3 (C) protein by western blot (B). Densitometry was measured by ImageJ. * P < 0.05 vs baseline.
A 1x EtOH Gavage

![Graph showing ALT (IU/ml) over hours for WT and cGAS-KO mice after 1x EtOH Gavage.](A)

B

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Densitometry fold increase

![Western blot analysis showing p-IRF3 and IRF3 for WT and cGAS-KO mice at different time points.](B)

C

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Densitometry fold increase

![Western blot analysis showing proCasp3, clvCasp3, and β-actin for WT and cGAS-KO mice at different time points.](C)
Mitochondrial DNA release as inception of disease

cGAS is an intracellular dsDNA sensor. cGAS is important in creating an anti-viral state in the presence of replicating viruses in the cytoplasm, leading to IFN-β through IRF3 activation. However, dsDNA from many sources, including host DNA, can activate cGAS, as it is not sequence dependent. We hypothesized that ethanol-induced mitochondrial damage, and subsequent mitochondrial DNA (mtDNA) release, may be responsible for activating cGAS. To test this, we isolated plasma from mice 2 and 4 hours post an ethanol binge. We measured for COX1 as a marker for mtDNA using digital droplet PCR (ddPCR). This approach allowed us to have highly sensitive detection method. Moreover, ddPCR yields absolute quantities of probe in our sample, rather than relative values compared to a reference gene, which is useful since plasma DNA is not expected to have unaltered nucleotide levels. We detected an increase in circulating mtDNA at 2 and 4 hours (Fig. 2.15A). To test if mtDNA was directly responsible for IRF3 phosphorylation, we had to make use of an in vitro system to manipulate endogenous levels of mtDNA. We used Huh7 cells, a transformed human hepatoma cell line. We incubated cells with ethidium bromide (0.2 µg/ml) and supplemented with 50 µg/ml uridine and 1 mM sodium pyruvate for 40 days to deplete mtDNA\textsuperscript{132}. We confirmed mtDNA depletion by Cytochrome C Oxidase I (COX1) and NADH Dehydrogenase Subunit 6 (ND6) mRNA (Fig 2.15B). We evaluated by microscopy whether ethidium bromide treatment reduced the number of overall mitochondria per cell. We found a comparable number of
mitochondria in WT and mtDNA-depleted Huh7 cells (Fig 2.15C), consistent with previous reports\textsuperscript{133,134}. Together, this suggested that prolonged ethidium bromide incubation led to reduction of mtDNA, but not in overall number of mitochondria. We then tested if \textit{in vitro} ethanol stimulation resulted in differences in phosphorylated IRF3 in these two cells. Stimulation with transfected dsDNA with poly(dA:dT)/LyoVec was used a positive control for cGAS-mediated IRF3 activation. We found an increase in ethanol-induced phosphorylated IRF3 in WT Huh7 cells. There was no detectable increase in phospho-IRF3 or cleaved Caspase-3 in the mtDNA-depleted cells (Fig 2.15D). This suggested that ethanol-induced cGAS-mediated activation of IRF3 and associated apoptotic signaling relied on mtDNA release into the cytoplasm. We hypothesize that apoptotic or necrotic cells from ethanol toxicity likely released their contents, including mtDNA, into circulation, a signal that was stronger at 4 hours than at 2 hours.
**Figure 2.15 Ethanol-induced mitochondrial DNA determines IRF3 phosphorylation**

WT mice were given a single ethanol dose. Plasma was isolated and evaluated for circulating mitochondrial DNA (mtDNA) by measuring COX1 mRNA by ddPCR (A). Huh7 cells were incubated with ethidium bromide (0.2 µg/ml), supplemented with 50 µg/ml uridine and 1 mM sodium pyruvate for 40 days to deplete mtDNA. mtDNA was confirmed by COX1 and ND6 mRNA by RT-PCR (B). 18s was used as non-mitochondrial mRNA control (C). WT and mtDNA-depleted Huh7 cells were evaluated for total mitochondria by confocal microscopy with MitoTracker staining (yellow), counterstained with DAPI (blue) (C). WT Huh7 and mtDNA-depleted Huh7 cells were stimulated with ethanol or transfected dsDNA (poly (dA:dT)/Lyovec), and evaluated for phospho-IRF3 and cleaved Caspase-3 by western blot (D).
A. Plasma mtDNA

<table>
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<th>4 hr</th>
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B. Mitochondrial mRNA

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C. Nuclear mRNA

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<tbody>
<tr>
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D. WT vs. mtDNA−

D. Huh7 hepatocyte cell line

E. Western Blot Analysis

- pIRF3
- IRF3
- cleaved Casp3
- β-actin
Accumulation of oxidized DNA in patients with Alcoholic Hepatitis

We hypothesized if ethanol consumption resulted in damaged mitochondria, perhaps this damage could accumulate in the liver. If so, it is likely to be present in the form of oxidized DNA. It has been reported that ethanol causes DNA damage and ROS-mediated oxidative stress\textsuperscript{135-137}. We therefore probed livers from patients with acute alcoholic hepatitis (AH) for 8-Hydroxy-2-deoxy-guanosine (8-OHdG), a marker for oxidative DNA damage\textsuperscript{138}. Compared to Normal controls, livers from disease patients with AH had increased 8-OHdG staining (\textbf{Fig. 2.16A}). The staining appeared to be cytoplasmic, or perhaps in mitochondria. To test this, we performed dual-immunofluorescent staining of a mitochondrial marker (COXIV) and 8-OHdG. Interestingly, the majority of the 8-OHdG staining was not localized near mitochondria (\textbf{Fig. 2.16B,C}). There was a quantifiable increase in 8-OHdG staining in diseased livers. We hypothesize that 8-OHdG staining may be the result of dead mitochondria or cytoplasmic mtDNA released into the cytoplasm.
**Fig. 2.16 Oxidized DNA increases in patients with alcoholic hepatitis**

Human livers from patients with acute alcoholic hepatitis and healthy normal controls were stained for 8-OHdG as a marker of oxidized DNA damage (A). Dual immunofluorescence staining for COXIV (green), 8-OHdG (red), and DAPI (blue) in livers from patients with alcoholic hepatitis and healthy controls (B). A binary mask was created in ImageJ to quantify 8-OHdG staining (C). Shown are livers from two representative patients per condition.
A

8-OHdG IHC: 200x; 600x

Normal

AH

B

COXIV 8-OHdG Merge 8-OHdG Binary

AH

AH

Normal

Normal

C

8-OHdG

Percent Positive Area

Normal  Alcoholic Hepatitis
Propagation of IRF3 activation through gap junctions

*IRF3 phosphorylation of naïve cells* in vitro

Inhibition of gap-junction communication limited the extent of the injury and protected against liver failure *in vivo*. Interestingly, in response to viral infection, cyclic dinucleotides were shown to spread through gap junctions\(^{50}\). Cyclic dinucleotide communication through gap junctions triggers STING and IRF3 activation in neighboring cells, thus amplifying the anti-microbial response. Gap junction communication through Cx32, the predominant connexin expressed by hepatocytes, can be blocked *in vitro* and *in vivo* using small-molecule inhibitor 2-aminoethoxydiphenyl borate (2APB), resulting in interference of intercellular Ca\(^{2+}\) ion flux\(^{139,140}\). We hypothesize that cyclic dinucleotides are small enough to be able to similarly cross hepatic hemi-channels and be blocked by 2-APB. We first tested this hypothesis with an *in vitro* assay for gap junction communication.

In a parachute assay\(^{141,142}\), functional gap junctions form between plated cells and a new donor cell, the ‘parachute’ cell, which is introduced to the culture. This occurs *in vitro* within two hours of plating parachute cells and can be visualized via real-time confocal microscopy in primary hepatocytes as calcein-AM, a gap junction-permeable dye, is diffused and transferred from parachute cells to naïve neighboring cells (*Fig. 2.17A*). Gap junction-mediated cytoplasmic transfer can be blocked *in vitro* with pre-treatment of 2APB for 15 minutes. (*Fig. 2.17B*). In a separate parachute assay, WT primary hepatocytes pre-incubated
with EtOH *in vitro* had phosphorylated IRF3 (**Fig. 2.17C,D** in orange).

Additionally, these donor cells were able to induce phosphorylation of IRF3 when plated on top of a monolayer of naïve cells (**Fig. 2.17 C,D** one recipient cell highlighted in white). This shows that propagation of ethanol-induced IRF3 signaling can be propagated via gap junctions *in vitro*.
Figure 2.17 Gap junctions propagate EtOH-induced IRF3 activation \textit{in vitro}

WT primary hepatocytes were plated (naïve); other (donor) cells were loaded with gap junction-permeable dye (calcein-AM, green). Cytoplasmic exchange was observed via confocal microscopy (A). Under same conditions, cells were pre-incubated with gap junction inhibitor 2APB for 15 min (B). In a separate experiment, WT primary hepatocytes were incubated in 200 mM EtOH for 2 hours (donor; highlighted with orange circle) and plated on hepatocyte monolayer (naïve; highlighted with white circle). Only naïve cells were labeled with DAPI (blue). All cells were fixed, stained for Actin (green) and p-IRF3 (red) and imaged (C). Same experiment as C, with images stacked on z-axis to create 3D image, rotated forward 45 degrees (D). White bar = 10 µm.
Gap junctions as therapeutic target in ALD

Given the zonal distribution of hepatocyte damage in ALD, we hypothesize that activation of IRF3 via gap junction-mediated cyclic dinucleotide contributes to the gradient of hepatocyte damage and subsequent amplification of cell death and inflammation in ALD. This hypothesis was supported in a previous report on drug-induced liver failure, where inhibition of Cx32 via 2APB was effective in preventing liver injury, inflammation and lethality from a lethal dose of APAP in mice\(^4^9\). Based on our observations that showed propagation of ethanol-induced signals through gap junctions resulting in IRF3 phosphorylation \textit{in vitro}, presumably cyclic dinucleotides, we hypothesized if hepatic gap junctions could be a therapeutic target in ALD. To test this, we administered a 10-day Lieber-DeCarli acute-on-chronic ethanol diet to WT mice, following a similar rationale as that used within the 4PBA and the cGAS experimental model. More specifically, this model would allow us to directly and comparably test if cGAS played a role in ALD pathogenesis in acute, chronic or acute-on-chronic ALD pathogenesis, while allowing us to tightly control time-specific dynamics, such as IRF3 phosphorylation. Detection of IRF3 phosphorylation would serve as clear indication of whether cGAS-deficient mice played a role or not in activating IRF3, and therefore served as a critical internal positive control in the WT control group. Some mice received daily intraperitoneal injections of 2APB. Mice that received 2APB had protection from liver injury and histological liver features (Fig. 2.18A,B). We then tested if the improved liver phenotype from 2APB was a result
of dampened IRF3 signaling. We found no detectable IRF3 phosphorylation in these mice at any of the time-points tested, with or without binge (Fig. 2.18C). We were surprised at how effective 2APB was at preventing ALD, since some hepatocytes would still be expected to metabolize ethanol in be exposed to hepatotoxic effects. We hypothesize that dampening the amplification of IRF3 activation and reduced inflammation allowed for the liver to maintain homeostasis in a manageable manner, even during ongoing acute and chronic ethanol exposure (Fig. 2.19).
Figure 2.18 Therapeutic inhibition of hepatic gap junction communication attenuates liver injury and IRF3 phosphorylation

WT mice were fed a 10-day acute-on-chronic Lieber-DeCarli ethanol diet containing 2APB (20 mg/kg/day). Some mice were sacrificed 4 and 9 hours after an ethanol binge. Liver injury was assessed by serum ALT (A) and H&E liver histology (B). Phosphorylation of IRF3 from whole-cell liver lysate of mice treated with or without 2APB (C).
10 days EtOH

A

10-day EtOH

WT

2APB

PF

0hr

4hr

9hr

+ 1x EtOH binge

serum ALT (U/L)

0

100

200

300

B

WT

2APB

PF

No Binge

4hr Binge

9hr Binge

C

+2APB, i.p.

10-day EtOH

hr: PF

No Binge

4hr Binge

9hr Binge

pIRF3

IRF3
Figure 2.19 Schematic of gap junction-mediated propagation of STING activation

Upon activation from mtDNA, cGAS secretes cyclic dinucleotide cGAMP, which binds and activates STING. cGAMP mean also travels via Cx32 gap junctions, spreading activation of STING and IRF3 to adjacent cells.
CHAPTER THREE: INFLAMMASOME SIGNALING

IL-1β maturation is a two-step process. LPS induces the pro-IL-1β gene expression but an additional, inflammasome-mediated signal is required for cleavage of pro-IL-1β protein to mature IL-1β that is released from inflammatory cells\textsuperscript{143}. We sought to identify the inflammasome sensor and the components released from damaged hepatocytes that serve as a second signal for IL-1β release using \textit{in vivo} and \textit{in vitro} models.

\textbf{NLRP3 as the inflammasome sensor in ALD}

\textit{NLRP3-deficient mice protected from inflammasome activation in ALD}

Our data indicated that uric acid and ATP released from damaged hepatocytes are required for activation of liver immune cells. The mechanism by which uric acid and ATP activate inflammation requires the NLRP3 inflammasome\textsuperscript{143}. Using the NLRP3, immune cells recognize endogenous "danger" signals, including ATP, uric acid and crystalline substances, and trigger activation of Caspase-1 and maturation of IL-1β\textsuperscript{71}. Therefore, we evaluated mice lacking NLRP3, and asked whether NLRP3 provides a mechanistic link between hepatocyte-derived danger signals, inflammasome activation and liver inflammation in ALD.

Deficiency of NLRP3 prevented ethanol-induced activation of the inflammasome in the liver, as indicated by the diminished levels of cleaved p10
The fragment of Caspase-1 (Fig. 3.1A) and cleaved IL-1β in the liver and in the serum (Fig. 3.1B,C), compared to WT mice. Active secreted IL-1β upregulates inflammatory cytokines, including TNF-α, promotes steatosis and sensitizes hepatocyte to cytotoxicity induced by TNF-α. Accordingly, lack of activation of inflammasome and IL-1β in NLRP3-deficient mice was accompanied by protection from liver inflammation, steatosis and damage, as indicated by the absence of ethanol-induced upregulation of TNF-α and IL-1β in the liver and in the serum (Fig. 3.1C), as well as improved findings on H&E and amelioration of ALT increase in the serum (Fig. 3.1D). The extent of protection from liver inflammation and injury in NLRP3-KO mice was comparable to that previously observed in mice deficient in Caspase-1/11. Since Caspase-1 is the effector molecule of inflammasomes, our data suggested that NLRP3 is a major activator of the inflammasome in ALD. At the time of these experiments, there was no commercially available mouse deficient in Caspase-1 only. It should be noted that although there have been reports of Caspase-11 involvement in the French-Tsukamoto model of ALD pathogenesis, I was unable to detect cleavage of Caspase-11 in any of the ALD models I have experimented with, such as single binge, 4-week chronic or in the 10-day plus binge model of ALD. Although an absence of Caspase-11 cleavage detection in my hands doesn’t rule out its potential role, this was sufficient evidence for me to assess that its role, at best, is at not greater than that of Caspase-1 in these models I tested.
Figure 3.1 Deficiency of NLRP3 ameliorates ethanol-induced liver inflammation

WT or NLRP3-KO mice were fed control (Pair-fed) or ethanol diet. After 4 weeks, we evaluated cleavage of Caspase-1 (A) and cleavage of IL-1β (B) by western blot. Levels of total IL-1β and TNF-α in circulation and liver lysate were assessed by ELISA (C). Liver damage was assessed by liver H&E histology and serum ALT (D). n = 7-15 mice per group. * < 0.05 versus baseline. Original magnification, 200x.
Endogenous sterile danger signals, ATP and uric acid, are increased in ALD

Given the effect of ethanol on mitochondrial function and metabolism of purine nucleotides, we studied the role of ATP and uric acid, two well-characterized activators of the inflammasome\textsuperscript{147}. First, we asked whether alcohol modulates the levels of uric acid and ATP. We evaluated uric acid and ATP in the serum and liver of WT mice after 4 weeks of Lieber-DeCarli ethanol or control diet feeding. We hypothesized that, if these DAMPs were to be accurately measured in circulation in mice, they were most likely to be detected \textit{in vivo} during peak levels of inflammation, i.e. after four-week ethanol diet, since at the time, the NIAAA model had not been thoroughly tested. We observed that liver damage caused by ethanol was accompanied by statistically significant increases in ATP and uric acid (\textbf{Fig. 3.2A}) levels in the serum and in the liver (\textbf{Fig. 3.2B}). This data indicated that liver damaged by ethanol releases metabolic danger signals, uric acid and ATP.
Figure 3.2 Endogenous inflammasome activators, ATP and uric acid, are increased in ALD

Wild-type mice were fed control (pair-fed) or alcohol Lieber-DeCarli diet. After 4 weeks, liver damage and uric acid and ATP in serum (A) and liver were evaluated (B). N= 15 (ethanol-fed); 5 (pair-fed). *P < 0.05 vs. baseline.
A

serum ALT

serum uric acid

serum ATP

B

liver uric acid

liver ATP
Hepatocyte-derived DAMPs as activators of NLRP3

To evaluate the source of increased ATP and uric acid in ALD, we exposed primary murine hepatocytes to ethanol or heat-shock (HS), and observed early release of ATP and uric acid into hepatocyte supernatants along with the development of hepatocyte injury (Fig. 3.3A,B). In contrast, ATP or uric acid was not detected in supernatants from liver mononuclear cells (LMNCs) exposed to ethanol or HS (Fig. 3.3C), indicating that increased ATP and uric acid detected in serum and livers of alcohol-fed mice was most likely derived from hepatocytes and not mononuclear cells. Treatment of LPS-primed LMNCs with uric acid crystals or ATP significantly increased the release of IL-1β protein indicative of inflammasome activation, and a similar increase in IL-1β was observed when LMNCs were treated with soluble uric acid (Fig. 3.3D). This data supported the notion that ATP and uric acid are released from damaged hepatocytes and are subsequently recognized as pro-inflammatory signals by LMNCs. This data indicated that ethanol-exposed hepatocytes release significant amounts of uric acid and ATP, and this observation was consistent with previous reports that uric acid and ATP are present in high concentrations in hepatocytes148,149.
Figure 3.3 Damaged hepatocytes, but not liver mononuclear cells, release endogenous metabolic activators of the inflammasome, ATP and uric acid

Primary murine hepatocytes were treated with 800 mM ethanol, and levels of LDH, indicating hepatocyte death, ATP and uric acid were evaluated in supernatants at indicated time points (A). Primary murine hepatocytes were cultured at 45°C (heat-shock) or 37°C (control) and levels of LDH, ATP and uric acid were evaluated in supernatants at indicated time points (B). Murine liver mononuclear cells (LMNCs) were treated with 800 mM ethanol, and levels of LDH, indicating cell death, ATP and uric acid were evaluated in supernatants at indicated time points (C). Murine LMNCs were cultured at 45°C (heat-shock) or 37°C (control) and levels of LDH, ATP and uric acid were evaluated in supernatants at indicated time points (C). LMNCs isolated from wild-type mice were pretreated with indicated doses of LPS. In some groups, ATP (5 mM) was added after 2 hours of LPS stimulation and supernatant was collected 1 hour afterward. In other groups, soluble uric acid (100 μg/mL) or monosodium urate crystals (MSU, 250 μg/mL) were added after 2 hours of LPS stimulation and supernatant was collected at 24 hours (D). Stimulations were performed in triplicates. *P < 0.05 vs. baseline.
Uric acid and ATP as second signal

Inhibition of ATP signaling in vivo

Our data indicating that exposure to ethanol increased circulating and liver ATP levels prompted us to ask whether ATP was required for activation of inflammasome and inflammation in chronic ALD. We used mice deficient in the P2rx7 receptor (purinergic receptor P2x, ligand-gated ion channel 7), which is activated by extracellular ATP\textsuperscript{71,150}. We observed that P2rx7-deficient mice were protected from alcohol-induced liver damage as indicated by improved liver histology on H&E staining and significantly lower serum ALT levels compared to wild-type mice (Fig. 3.4A,B). Ethanol-induced increase in the inflammatory cytokine, TNF-α (Fig. 3.4C), was prevented in the serum and liver of mice deficient in ATP signaling compared to WT mice. In contrast to WT mice, there was no ethanol-induced increase in serum IL-1β in P2rx7-deficient mice (Fig. 3.4D). IL-1β in the serum is present predominantly in the bioactive form, suggesting a lack of inflammasome activation in the absence of ATP signaling. Indeed, the deficiency of P2rx7 prevented alcohol-induced activation of the inflammasome in the liver, demonstrated by undetectable levels of p10, the cleavage product of the inflammasome effector molecule, Caspase-1 (Fig. 3.4E). This data supported the hypothesis that ATP plays an important role in activation of the inflammasome as well as in development of liver inflammation, steatosis and injury in ALD.
Figure 3.4 ATP signaling is required for inflammasome activation and liver inflammation in ALD

WT or P2rx7-KO mice were fed control (pair-fed) or Lieber-DeCarli alcohol diet. After 4 weeks, liver damage was assessed by liver H&E histology (A) and serum ALT (B). We evaluated levels of TNF-α in the serum and in the liver (C) and IL-1β in the serum by ELISA (D). We evaluated cleavage of Casp-1 in the liver by western blot (E). N= 7-15 (ethanol-fed, per genotype); 5 (pair-fed, per genotype). Numbers in the graphs indicate P values. *P < 0.05 vs. baseline. Original magnification, 200x.
**Targeting uric acid in vivo**

Our data indicated that exposure to ethanol increases the release of uric acid from the damaged liver. To evaluate whether uric acid is required for liver inflammation in ALD, we used two previously described strains of transgenic (Tg) mice overexpressing uricase (urate oxidase, UOX), an enzyme that degrades uric acid\textsuperscript{151,152}. In the first strain, referred to as secreted uricase (ssUOX)-Tg, uricase is secreted into the extracellular fluids, depleting uric acid from the interstitial fluids, tissues and serum\textsuperscript{151}. In the second strain, referred to as intracellular uricase (intUOX)-Tg, uricase is targeted to peroxisomes and reduces intracellular pools of uric acid. After intUOX-Tg cells die and uricase is released, the enzyme also depletes uric acid from the environment of dead cells\textsuperscript{151}. Both strains of Tg mice showed absence of alcohol-induced increase of uric acid in the serum and in the liver (Fig. 3.5A), compared to WT mice. The intUOX-Tg demonstrated a trend towards lower baseline levels of uric acid in the serum and in the liver compared to ssUOX-Tg mice, but the difference was not significant (Fig. 3.5A).

We observed that depletion of uric acid in ssUOX-Tg or intUOX-Tg mice provided significant protection from liver damaged as evaluated by H&E liver histology and serum ALT (Fig. 3.5B,C). Importantly, uric acid-deficient mice showed lack of alcohol-induced increase of IL-1β in the serum (Fig. 3.5D), which indicated decreased inflammasome activation in absence of uric acid signaling. Indeed, further analyses showed that depletion of uric acid in ssUOX-Tg or
intUOX-Tg mice prevented alcohol-induced activation of inflammasome in the liver, demonstrated by absent cleavage of the inflammasome effector molecule, Caspase-1, with more pronounced repression of Caspase-1 cleavage in intUOX-Tg, compared to ssUOX-Tg mice (Fig. 3.5E). This data supported our hypothesis that uric acid is involved in activation of inflammasome and development of liver inflammation, steatosis and injury in ALD.
Figure 3.5 Uric acid is required for inflammasome activation and liver inflammation in ALD

WT, ssUOX-Tg or intUOX-Tg mice were fed control (pair-fed) or alcohol Lieber-DeCarli diet. After 4 weeks, we evaluated levels of uric acid in serum and in the liver (A). Liver damage was assessed by liver H&E histology (B) and serum ALT (C). Levels of IL-1β in were evaluated in the serum (D). We evaluated cleavage of Caspase-1 in the liver (E). N= 6-9 (ethanol-fed, per genotype); 4-5 (pair-fed, per genotype). Numbers in the graphs indicate P values. *P < 0.05 vs. baseline. Original magnification, 200x.
**A** Serum uric acid and liver uric acid levels in wild type (WT) and ssUOX-Tg mice. Significance levels are indicated by *P* values.

**B** H&E staining of liver tissue from WT and ssUOX-Tg mice fed pair-fed or EtOH diet for 28 days. Images show typical histological findings associated with ethanol consumption.

**C** Serum ALT levels over time in WT and ssUOX-Tg mice fed pair-fed or EtOH diet. Global differences are indicated by *P* values.

**D** Serum IL-1β levels in wild type, ssUOX-Tg, and intUOX-Tg mice fed pair-fed or EtOH diet. Significance levels are indicated by *P* values.

**E** Immunoblotting of Casp-1 and β-actin expression in wild type and intUOX-Tg mice fed pair-fed or EtOH diet. Densitometry analysis shows fold-increase in Casp-1 p10 expression compared to β-actin.
Therapeutic intervention with allopurinol or probenecid

Finally, we asked whether uric acid and ATP could represent potential therapeutic targets in alcoholic steatohepatitis. To deplete uric acid, we evaluated two anti-gout medications used in humans: allopurinol, an inhibitor of xanthine oxidase, an enzyme that synthesizes uric acid, and probenecid, a compound promoting renal excretion of uric acid.

Treatment with allopurinol significantly attenuated alcohol-induced histological changes in the liver on H&E staining and reduced serum ALT (Fig. 3.6A,B). This protection from alcohol-induced liver injury was consistent with significant, albeit incomplete, reduction in alcohol-induced cleavage of Caspase-1 (Fig. 3.6C), whereas full prevention of Caspase-1 cleavage in the liver was observed when mice were treated with probenecid (Fig. 3.6C). Allopurinol-treated mice also demonstrated complete protection from TNF-α increase in the serum and in the liver (Fig. 3.6D). Amelioration of alcohol-induced damage on histology was consistent with a previous report153. Importantly, daily intragastric administration of allopurinol or probenecid significantly reduced levels of uric acid in the serum of alcohol-fed mice, and treatment with allopurinol decreased baseline levels of serum uric acid to almost non-detectable levels (Fig. 3.6E). However, treatment with probenecid provided superior protection from ALD. This additional protective effect implied that mechanisms beyond promotion of uric acid excretion may be responsible for the protective effect of probenecid. It has been reported that probenecid inhibits pannexin-1 channels154, which play an
essential role in ATP signaling\textsuperscript{155}. Thus, the superior protective role of probenecid in ALD was likely explained through the promotion of uric acid excretion as well as inhibition of ATP signaling, indicating a synergistic effect of uric acid and ATP on inflammasome activation in ALD.
Figure 3.6 Pharmacologic depletion of uric acid and inhibition of ATP signaling prevents alcoholic steatohepatitis and fibrosis

WT mice treated with daily intragastric doses of vehicle (PBS), allopurinol or probenecid (100 mg/kg body weight) were fed control (pair-fed) or alcohol Lieber-DeCarli diet. After 4 weeks, liver damage was assessed by H&E histology (A) and serum ALT (B). We evaluated liver cleavage of Caspase-1 by western blot (C) and levels of liver TNF-α in the serum by ELISA (D). We evaluated the levels of uric acid in the serum (E). N= 9-10 mice (ethanol-fed, per treatment); 3-5 mice (pair-fed, per treatment). Numbers in the graphs indicate P values. *P < 0.05 vs. baseline. Original magnification, 200x.
**serum Uric Acid**

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**pair-fed vs. EtOH-fed**

- Vehicle vs. allopurinol: $P = 0.008$
- Vehicle vs. probenecid: $P < 0.001$
- Allopurinol vs. probenecid: $P < 0.001$

**immunoblotting**

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**serum Uric Acid**

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<td>mmol/L</td>
<td>0.000</td>
<td>0.007</td>
<td>0.023</td>
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Overall, this data suggested the presence of cross-talk between alcohol-damaged hepatocytes and liver immune cells and demonstrated that inflammatory activation of LMNCs is augmented by hepatocyte-derived uric acid and ATP. This signaling axis is dependent on a feed-forward induction via inflammasome activation and release of IL-1β, reflecting the dependency of IL-1β induction on autocrine loop mediated by IL-1 receptor\textsuperscript{145,156,157}.

**Recovery with Anakinra**

Impaired liver regeneration correlates with poor survival in patients with severe acute alcoholic hepatitis (AH)\textsuperscript{158}. The inability of patients with AH to restore fully functional hepatocyte mass and function has been reported in multiple clinical studies,\textsuperscript{158,159} but the mechanisms of this phenomenon are yet to be elucidated. Studies using mouse models of ALD have focused predominantly on the pathogenesis of ALD (reviewed in ref.\textsuperscript{160,161}) but have not addressed hepatocyte regeneration during the recovery phase. The aim of this study was to propose a mouse model that would provide a tool to study liver recovery after exposure to chronic ethanol. We also used this model to investigate whether liver recovery can be facilitated by inhibition of IL-1, a cytokine crucially involved in the pathogenesis of ALD through NLRP3 activation of the inflammasome in mice\textsuperscript{64,162,163}.

In our model, we attempted to mimic human drinking patterns of acute-on-chronic ethanol exposure in alcoholic patients. WT mice were fed the Lieber-
DeCarli ethanol diet for 4 weeks. To enhance liver injury, mice received 3 acute doses of ethanol via an intragastric probe during the last 3 days of ethanol feeding (Maximal Injury) (Fig. 1.7). Subsequently, all mice were transitioned to the control diet with no ethanol and randomly assigned to daily treatment with Anakinra (IL-1Ra, an inhibitor of IL-1 signaling) or saline (Recovery phase), both delivered via intraperitoneal injections. Mice were sacrificed at baseline, at the point of maximum injury, and during the recovery phase, 1, 2, 3, 4 and 7 days after transition to control diet.
Figure 3.7 Design of murine model of alcoholic hepatitis

WT mice were treated with ethanol diet for 4 week and received three intragastric gavages of EtOH (5 g/kg) during the last 3 d of ethanol feeding ("Max. Injury," red). After day 28, all mice were switched to control diet, and daily treatment with Anakinra or saline was initiated (Recovery Phase, blue). Baseline (green) refers to mice sustained on iso-caloric control liquid diet for the duration of the feeding (week 5, day 7).
Lieber-DeCarli chronic ethanol (5% vol/vol)
Control liquid diet
Acute ethanol gavage (5 g/kg)
Anakinra (25 mg/kg) or saline, i.p.

Acute EtOH x3
Anakinra or saline

Day:
1 7 14 21 28 1 2 3 4 5 6 7

Week 1 Week 2 Week 3 Week 4 Week 5

Chronic Ethanol Control Diet

Sacrifice: BASELINE
MAX. INJURY
RECOVERY PHASE
Hepatocyte Ballooning

At the point of ethanol-induced maximum injury, H&E staining showed large inclusions of fat, some hepatocyte ballooning, and mild inflammatory infiltrate (Fig. 3.8A). In the recovery phase, ballooning slowly resolved within 72 hours (Fig. 3.8B), and scattered foci of inflammatory cells surrounding dead hepatocytes appeared within 24 hours but disappeared within 48 hours (Fig. 3.8C). Compared to saline-treated mice, the extent of liver inflammatory cell infiltration was significantly lower in mice treated with Anakinra. No significant differences were observed in the extent of ballooning. As ballooning provides only semi-quantitative assessment of hepatocyte damage, we used serum ALT for quantitation of hepatocyte damage. The mean ALT level at the point of maximal injury was 250 U/L (Fig. 3.8D), which is approximately two times higher compared to levels we observed in previous studies in mice on Lieber-DeCarli ethanol diet without acute ethanol binge\textsuperscript{92,110,164}. ALT levels normalized by day 4 in saline-treated mice and by day 2 in mice treated with Anakinra, indicating that inhibition of IL-1 signaling facilitates recovery from liver injury (Fig. 3.8D).
Figure 3.8 Treatment with Anakinra facilitates recovery from acute-on-chronic alcoholic liver injury

Liver injury was analyzed using H&E staining (A) and hepatocyte death was quantitatively evaluated using serum ALT (B). Semi-quantitative scoring was performed for histological features of hepatocellular ballooning (0-2) (C) and lobular inflammation (0-2) (D). n=3–5 per time point and treatment. Numbers in graphs indicate P-values. *P≤.05 vs baseline
A. H&E, 200x  
    inset: 400x  

Recovery Phase:  
<table>
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<tr>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 7</th>
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<tr>
<td>Saline</td>
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<tr>
<td>Anakinra</td>
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B. Ballooning  
C. Inflammation  
D. ALT

- Saline  
- Anakinra

EIOH diet 28 days + acute EIOH x3  
Conversion to control diet + daily Anakinra or saline

EIOH diet 30 days + acute EIOH x3  
Conversion to control diet + daily Anakinra or saline

EIOH diet 30 days + acute EIOH x3  
Conversion to control diet + daily Anakinra or saline
We further evaluated the role of Anakinra on inflammation by probing for neutrophil liver infiltration using myeloperoxidase (MPO) immunohistochemistry. We found levels of MPO staining had rapidly normalized by day 1 of recovery phase in mice treated with Anakinra and remained low, while saline-treated mice maintained elevated levels as late as day 4 (Fig 3.9A,B).

Lastly, Oil-red-O staining of the liver, that was used to evaluate the accumulation of fat, showed significant steatosis at the point of maximal injury (Fig 3.9C). In the recovery phase, fat droplets disappeared and steatotic score dropped within 24 hours (Fig 3.9D). We observed rapid regression of steatosis during the recovery phase which was so fast that we could not evaluate the role of Anakinra in this scenario.
Figure 3.9 Treatment with Anakinra facilitates recovery from acute-on-chronic alcoholic liver inflammation and steatosis

Neutrophil liver infiltration was assessed by MPO immunohistochemistry (A) with LPS-injected mouse liver (0.5 mg/kg, 12 h) used as positive control (Pos. Control), and positive cells per visual field were quantified, average of five images per sample (B). Steatosis was evaluated by Oil-red-O staining (C) and scored semi-quantitatively on a scale from 0-3 (D). n=3–5 per time point and treatment. Numbers in graphs indicate P-values. *P≤.05 vs baseline
Hepatocyte regeneration

To evaluate liver regeneration, we stained livers for Ki67, a marker expressed by proliferating cells but absent in quiescent cells. Livers harvested from mice at baseline and at the point of maximal injury showed minimal Ki67 signal, indicating minimal proliferative activity and there was no significant difference between these two time-points (Fig. 3.10A). Compared to the point of maximal injury, increased Ki67 nuclear staining was present in hepatocytes from saline-treated mice on days 1 and 2 of recovery. Furthermore, the increase in Ki67 was even greater in mice treated with Anakinra (Fig. 3.10A,B). Image analysis showed a significant increase in the proportion of Ki67-positive hepatocytes on days 1 and 2 of recovery, and this proportion was significantly higher in mice treated with Anakinra compared to saline treatment.
Figure 3.10 Treatment with Anakinra enhances liver regeneration from acute-on-chronic alcoholic liver injury

WT mice were treated with ethanol diet for 4 weeks and received three intragastric gavages of EtOH (5 g/kg) during the last 3 d of ethanol feeding (Max. Injury). After day 28, all mice were switched to control diet, and daily treatment with Anakinra or saline was initiated (Recovery Phase). Liver regeneration was analyzed using Ki67 (A). Quantification of Ki67 signal was performed using ImageJ (B). n=3–5 per time point and treatment. Numbers in graphs indicate P-values. *P≤.05 vs baseline
A.

Ki67, 200x
inset: 400x

Recovery Phase:

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<th>Day 1</th>
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B.

Ki67+ nuclei

EIOH diet 28 days + acute EIOH x3
conversion to control diet + daily Anakinra or saline
We further tested our hypothesis by evaluating cyclin D1 levels in the liver. Cyclin D1 is a nuclear protein required for progression through the G1 phase of the cell cycle. Cyclin D1 liver immunohistochemistry staining in murine liver sections 24 hours post partial hepatectomy served as a positive control (Fig. 3.11A). We found that hepatocellular size increased after partial hepatectomy, with distinct nuclear and cytoplasmic expression in controls, while ethanol administration resulted in a different pattern of expression similar to that observed with Ki67 staining. Mice treated with Anakinra had increased levels of cyclin D1 protein expression per visual field compared to saline-treated controls (Fig. 3.11A,B). This data indicated that inhibition of IL-1 signaling facilitates liver regeneration in ethanol-induced liver injury. The proportion of Ki67-positive hepatocytes significantly decreased by day 3 and returned to baseline by day 4 of recovery both in the Anakinra and saline treated groups, while Anakinra-treated mice had consistently higher levels of cyclin D1 throughout the recovery phase.
Figure 3.11 Treatment with Anakinra enhances hepatocyte regeneration from acute-on-chronic alcoholic liver injury

WT mice were treated with ethanol diet for 4 weeks and received three intragastric gavages of EtOH (5 g/kg) during the last 3 d of ethanol feeding (Max. Injury). After day 28, all mice were switched to control diet, and daily treatment with Anakinra or saline was initiated (Recovery Phase). Liver regeneration was analyzed using cyclin D1 (A), with murine liver 24-hours post partial hepatectomy used as positive control (Pos. Control). Cyclin D1 positive cells were counted per visual field, average of five images per sample (B). n=3–5 per time point and treatment. Numbers in graphs indicate P-values. *P≤.05 vs baseline
A.

**CyclinD1, 200x**

Inset: 400x

**Recovery Phase:**

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<tr>
<td>Anakinra</td>
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<td><img src="image10.jpg" alt="Image" /></td>
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</table>

B.

**Cyclin D1+**

- **saline**
- **Anakinra**

- E1OH diet 28 days
- Conversion to control diet
- + acute E1OH x3
- + daily Anakinra or saline

---

Nuclei pos. cell count per visual field
Finally, to assess liver size, we analyzed liver mass as a function of bodyweight of mice at the time of sacrifice (Fig. 3.12). We found that saline-treated mice had increased liver size at day 2, the same time-point associated with increased hepatocellular ballooning (Fig. 3.8A,B). Importantly, Anakinra-treated mice had an increase in liver size at day 7 of the recovery phase, compared to the saline-treated controls. These findings correlate positively with the increased enhanced hepatocyte regeneration of mice treated with Anakinra (Fig. 3.10, 3.11).
Figure 3.12 Liver-to-bodyweight ratios after acute-on-chronic alcoholic liver injury

WT mice were treated with ethanol diet for 4 weeks and received 3 intragastric gavages of EtOH (5 g/kg) during the last 3 days of ethanol feeding. After day 28, all mice were switched to control diet, and daily treatment with Anakinra or saline was initiated. Baseline refers to mice sustained on iso-caloric control liquid diet for the duration of the feeding (Week 5, day 7). Mice and excised livers were weighed at the time of sacrifice; data presented as fold increase of liver/mouse mass (A). n = 3–5 per time point and treatment. *P ≤ 0.05 vs. baseline.
Liver to Bodyweight

Fold Change

- salineline
- Anakinra

Baseline max. injury day 1 day 2 day 3 day 4 day 7

EtOH diet 28 days + acute EtOH x3 conversion to control diet + daily Anakinra or saline

0.0003 0.04 0.05
Pyroptosis

When immune cells have inflammasome activation, IL-1β and IL-18 secretion were not the only signaling molecules observed being excreted. Interestingly, release of ATP, HMGB1 and IL-1α release was also observed. HMGB1 and IL-1α are particularly interesting because they fall under the category of alarmins – DAMPs that can have a dual role, with or without post-translational modifications, when secreted. Although inflammasome activation requires certain caspase activity, such as Caspase-1 or Caspase-11, it wasn’t known for some time how exactly Caspase-1 activation was eventually followed by cell death. This regulated, necrotic-like cell death dependent on inflammasome activation is called pyroptosis, resulting in DAMPs release through cell lysis. Pyroptosis, however, has not been identified in the context of ALD. A recent study created a synthetic model of inflammasome signaling in the liver through genetic manipulation with constitutively active NLPR3 in hepatocytes. This resulted in a fibrotic phenotype, along with pyroptosis of hepatocytes, as one would expect. The same group has also shown an important role of NLRP3 in progression of non-alcoholic steatohepatitis (NASH), however, this study did not probe for presence of pyroptosis, and the inflammasome activation was not shown to be localized to hepatocytes, but rather in total liver.

In our first experiment tracking total number of liver macrophages over a period of seven days of ethanol administration, we had identified an initial
decrease in macrophage numbers in the liver of adoptive transfer of GFP+ macrophages upon initial ethanol exposure, followed by recruitment of more macrophages. (Fig. 3.13A-C). One possible explanation was that macrophages were initially leaving the liver, possibly to communicate with other immune cells for recruitment, and then returning after several hours or days. However, the most likely explanation for these observations was that due to inflammasome activation, macrophages were undergoing pyroptosis, and through IL-1β signaling and sustained DAMP release from hepatocytes, more macrophages were recruited. We therefore hypothesized that pyroptosis was taking place in liver macrophages upon inflammasome activation after sustained ethanol exposure. To test this, we isolated Kupffer Cells and overall liver mononuclear cells (LMNC) from ethanol-fed and pair-fed mice after acute-on-chronic ethanol administration. We found that there was a decrease in CD45+ cells over all, especially in F4/80, CD11b+ cells. Similarly, we observed a decrease in liver macrophage numbers after an ethanol binge after ten days of chronic ethanol administration (Fig. 3.14A-B).
Figure 3.13 Ethanol exposure results in initial decrease of macrophages with subsequent increase in the liver

Mice overexpressing GFP in BM-derived cells were fed Lieber-DeCarli diet and sacrificed at indicated time points. Livers were perfused, LMNCs were isolated, and analyzed via Flow Cytometry as indicated in the gating strategy (A). Percentage of GFP-positive (i.e. BM-derived) inflammatory cells in the liver was determined in the CD11b+/F4/80+ population of liver immune cells (B) and (C). Cells plotted by side-scatter-area (SSC-A) vs. GFP expression (FITC-A).
A. **BMT protocol**
- C57Bl/6 WT mice
- whole body irradiation
- 1 day
- Transplantation of BM from GFP-transgenic mice
- 5 weeks
- Feeding with EtOH diet 0, 1, 3 or 7 days
- Isolation of liver mononuclear cells

**FACS gating protocol**
- Liver mononuclear cells
- Live cells Singlets
- CD11b\(^+\)F4/80\(^+\) cells
- GFP\(^+\) cells

B. **CD11b\(^+\) F4/80\(^+\) GFP\(^+\)**
- Baseline (day 0)
- EtOH 1 day
- EtOH 3 days
- EtOH 7 days

C. **GFP\(^+\) cells**
- % within CD11\(^+\) F4/80\(^+\) cells
- Day: 0, 1, 3, 7
Figure 3.14 Kupffer cell numbers decrease after acute-on-chronic ethanol administration

WT mice were fed a Lieber-DeCarli diet for 10 days and sacrificed 9 hours after an ethanol binge (5 g/kg). Livers were perfused, and LMNCs were isolated. Cells were gated for live, CD45\(^+\), CD11b\(^+\) and F4/80\(^+\) expression. Kupffer cells, CD45\(^+\) F4/80\(^{\text{high}}\) CD11b\(^+\) (A); infiltrating macrophages, CD45\(^+\) F4/80\(^{\text{mid}}\) CD11b\(^+\) (B); neutrophils, CD11b\(^+\) Ly6G\(^+\) (C); and ALT vs neutrophil percent plotted.
To test if this decrease in liver macrophages was caspase-dependent pyroptosis, we tested inhibition of Caspase-1 activation in a model of ALD. We hypothesized that caspase inhibitors would be effective in preventing inflammasome activity and subsequent pyroptosis. We administered mice with either a pan-Caspase inhibitor, z-VAD-fmk, an FDA-approved Caspase-1 inhibitor, VX-765, or a necroptosis inhibitor, Necrostatin-1, to target multiple pathways of programmed cell death in parallel.

VX-765 (Belnacasan) is an orally absorbable pro-drug of VRT-043198 effective against Caspase-1 and -4. Z-VAD-fmk is a cell-permeable inhibitor of caspase proteases, including Caspase-1, -3, -8, and -9, all of which have been implicated in ALD. Finally, Necrostatin-1 is a RIPK1 inhibitor, needed for necroptosis activation.

We detected a significant decrease in liver injury as measured by serum ALT in mice treated with z-VAD-fmk and VX-765 (Fig. 3.15), suggesting that Caspase-1 as well as other caspases play a role in furthering ethanol-induced liver injury. An absence of change in ethanol-induced liver injury after Necrostatin-1 administration suggests that cell death from acute effects are not related to necroptosis.
Figure 3.15 Protection in liver injury from Caspase inhibition

Mice were given an acute-on-chronic ethanol diet and sacrificed 4 or 9 hours post ethanol binge. Pair-fed mice were sacrificed 9 hours post a sugar binge. During the experiment, mice were injected with a pan-Caspase inhibitor (z-VAD-fmk), a Caspase-1 inhibitor (VX-765), or a necroptosis inhibitor (Necrostatin-1) or vehicle. Serum ALT was analyzed as marker for liver injury (A).
Serum ALT

**Pair-fed**
- **EtOH +4h Binge**
- **EtOH +9h Binge**

Vehicle, z-VAD-fmk, VX-765, Necrostatin-1
Gasdermin D as cell executioner

In 2015, gasdermin D (Gsdm D) was first identified as the executioner of pyroptosis. Upon cleavage by Caspase-11 or Caspase-1, gasdermin D is cleaved, forming a pore in the plasma membrane of the cell\textsuperscript{186,187}. This quickly triggers the necrotic cell death previously identified. Interestingly, the gasdermin family of proteins may have a variety of roles associated with apoptosis\textsuperscript{188}. We hypothesized that gasdermin D may play a role in directing LMNCs to undergo pyroptosis upon ethanol-induced inflammasome activation. To test this, we probed for presence of cleaved gasdermin D protein in liver lysate using an antibody specific for the N-terminal amino acids 126-138\textsuperscript{189}. We found an increase in cleaved gasdermin D in liver 9 hours after a single binge as part of acute-on-chronic ethanol administration (10 days) (\textbf{Fig. 3.16A,B}). To test for gasdermin D cleavage in a model of alcoholic sepsis, we administered 10 days of Lieber-DeCarli ethanol diet followed by an injection of low dose LPS (0.5 mg/kg). Interestingly, we found that a single injection of LPS did not lead to gasdermin D cleavage, but did accentuate this signal if the LPS injection was administered after chronic ethanol (\textbf{Fig. 3.1C,D}). The timing of gasdermin D cleavage, peaking at 9 hours post binge, correlates with the peak of inflammasome activity, measured 4 hours post binge. It would be expected that cleavage of IL-1\textbeta by Caspase-1 would be subsequently followed by gasdermin D cleavage.
Figure 3.16 Gasdermin D cleavage in ALD

Mice were given a 10 days Lieber-DeCarli ethanol diet and sacrificed 4 or 9 hours post ethanol binge. Pair-fed mice were sacrificed 9 hours post a sugar binge. Mice were sacrificed, whole cell liver lysate was analyzed via western blot for N-terminal gasdermin D cleavage (A) and cleaved gasdermin D (Gsdm D) band was quantified using ImageJ (B). WT mice were given Lieber-DeCarli diet for 10 days and some mice received a low dose LPS injection (0.5mg/kg) and sacrificed 9 hours post LPS injection. Pair-fed mice were sacrificed 9 hours post a sugar binge. Mice were sacrificed, whole cell liver lysate was analyzed via western blot for N-terminal gasdermin D cleavage (C) and cleaved gasdermin D band was quantified using ImageJ (D).
A.

B.

C.

D.
Extracellular ASC aggregates

Based on our findings that pyroptosis of LMNCs indeed occurs in ALD through gasdermin D, which is required for IL-1β secretion\textsuperscript{187}, we sought to investigate the role of extracellular apoptosis-associated speck-like protein containing a CARD) (ASC) and its potential association with pyroptosis. Upon inflammasome assembly, ASC (previously known as PYCARD) proteins are phosphorylated and polymerize into an enormous intracellular aggregate, allowing for the recruitment and activation of Caspase-1\textsuperscript{190-194}. There have also been multiple reports of active Caspase-1 fragments in cell supernatants\textsuperscript{195-204}. These reports suggest that a fully active inflammasome assembly is released by necrotic cells upon inflammasome activation. Of particular interest, however, is ASC itself, which is released and remains active extracellularly\textsuperscript{205,206}. Extracellular inflammasome components have been previously identified in the serum of patients with cryopyrin-associated periodic syndromes (CAPS), associated with abnormally high levels of inflammasome signaling due to gain-of-function mutations in NLRP3\textsuperscript{205}.

It is possible that some inflammasome components released by pyroptotic cells are further contributing to the inflammatory cascade by being internalized by liver macrophages or other cell types such as hepatic stellate cells\textsuperscript{207,208}, and even hepatocytes, which have been shown to, at the very least, express components of inflammasome signaling\textsuperscript{171,209}. Given these observations, we
hypothesized that ethanol-induced inflammasome activation results in ASC speck deposits in the liver, furthering the inflammatory cascade.

Using an ELISA for ASC, we found an increase in ASC protein circulating in patients with acute AH compared to healthy controls. We confirmed our results via immunoprecipitation of ASC analyzed via western blot from plasma of AH patients. To gain a better insight as to the cell type specificity and overall expression pattern of ASC in the liver, we analyzed liver sections of cirrhotic patients with acute AH via immunohistochemistry for ASC protein. We found extremely high levels of ASC protein (Fig. 3.17). We expected to see low levels of ASC expression in healthy controls since immune cells will express non-polymerized ASC under normal, unstimulated conditions. Interestingly, ASC expression in diseased patients showed a consistent pattern of massive aggregates, most of which appear to be extracellular in nature. This created a spotted pattern through the liver, resembling a phenotype I'll describe as *Camelopardalis maculae*, or spots of a giraffe. We probed livers from ten different diseased patients and this pattern was present in all of them, while being consistently absent in all normal livers tested. We tested for specificity of the staining by testing three different anti-ASC antibodies from different manufacturers, as well as testing non-specific binding of the antibody by using livers from ASC-knockout mice.
Figure 3.17 ASC aggregates in livers from patients with AH

Normal and cirrhotic livers from patients with AH were probed for presence of ASC protein via immunohistochemistry. Numbers above each box correspond to a patient identifying number. Magnification at 400x.
ASC Immunohistochemistry: 400x

Normal

Alcoholic Hepatitis
Surprisingly, ASC aggregate formation in diseased liver was not exclusive to patients with AH. We tested five cirrhotic livers from patients with non-alcoholic steatohepatitis (NASH) and all five were also positive for large deposition of ASC protein, showing the same *Camelopardalis maculae* pattern observed in patients with AH (Fig 3.18). Although the pathogenic role of inflammasome signaling in NASH (reviewed here) was once controversial, with one prominent study reporting that NLRP3 has protective effects, several recent reports have implicated NLRP3 in the pathogenesis of NASH. Our findings correlate with the latter group, suggesting an important role of ethanol-induced pyroptosis-mediated extracellular ASC in an inflamed, fibrotic liver.
Figure 3.18 ASC aggregates in livers from patients with NASH

Normal and cirrhotic livers from patients with NASH were probed for presence of ASC protein via immunohistochemistry. Numbers above each box correspond to patient identifying number. Magnification at 400x.
ASC IHC
Non-alcoholic Steatohepatitis
NLRP3 inhibition using MCC950

The identification *Camelopardsalis maculae* phenotype in AH patients is important not just for the extent of expression in diseased patients, but also because of what this implies in term of future therapies. As mentioned earlier, the Defeat Alcoholic Steatohepatitis (DASH) U01 clinical trial for severe AH incorporates the use of IL-1Ra, which inhibits IL-1 signal propagation by inhibition of IL-1R in the presence of Caspase-1 activity and cleaved IL-1β. However, using IL-1R as a therapeutic target implies permissible inflammasome activity, with concomitant pyroptosis, DAMPs release and ASC aggregate formation in the liver. IL-1Ra therapy therefore is best at preventing the inflammatory cascade that results from feed-forward mechanisms from inflammasome signaling in the diseased state, but only if driven through IL-1 signaling, but not by the branching-off pathways. While IL-1Ra has shown promising results in animal models in treatment of ALD, it seems perhaps prudent to consider an alternate approach to target inflammasome signaling upstream of its activation. For this reason, we decided to test the efficacy of MCC950, an NLRP3-specific small-molecule inhibitor, as a potential novel therapy.

In an acute model of early ALD, we administered three ethanol binges over a period of three days. Mice were sacrificed 4 hours after the final binge, for peak inflammasome signal in the liver, but not for overall liver injury. Mice that were pre-treated with MCC950 had reduced levels of serum ALT (*Fig 3.19A*). At this time-point, where mice were sacrificed 4 hours post the final binge, the
morphological features in liver histology were not drastically different (Fig. 3.19B). We did detect a significant reduction in liver inflammasome activation, with reduced levels of Caspase-1 and IL-1β cleavage (Fig. 3.19C). We decided to test this NLRP3 inhibitor in a more robust model of liver injury using the standard 4 week Lieber-DeCarli model of chronic ALD. However, we tested MCC950 as a potential therapy to an already established injured liver, not as a preventative approach where the compound is administered through the feeding. We found that mice treated with MCC950 had an effective reduction in liver injury compared to their ethanol-treated group (Fig 3.19D). This suggests that NLPR3 inhibition via a small molecule inhibitor is an effective therapy in a murine model of ALD.
Figure 3.19 *In vivo* inhibition of ethanol-induced NLRP3 inflammasome with MCC950

WT mice were administered three doses of acute ethanol. MCC950 was administered at a dose of 5 mg/kg/day, i.p., to some mice 30 min. prior to ethanol binge. Liver injury was assessed by serum ALT (A) and H&E liver histology (B). Efficacy of inhibition of inflammasome signaling was assessed by measurement of Caspase-1 and IL-1β cleavage in liver by western blot and quantified by ImageJ (C). WT mice were fed a 4 week Lieber-DeCarli ethanol diet. After two weeks, some mice received daily injections of MCC950. At the conclusion of the experiment, liver injury was assessed by serum ALT (D). Acute ethanol model: n = 3–5 per time point and treatment; chronic ethanol model: n = 6-10 mice. *P ≤ 0.05 vs. baseline; ***P ≤ 0.0001 vs. DMSO-treated ethanol group.
3x binge acute ethanol

A

Serum ALT

B

3x Sugar 3x EtOH

DMSO MCC950

C

i.p.: DMSO MCC950
Sugar EtOH Sugar EtOH

Casp1p10
β-actin
clv IL-1β
β-actin

4wk chronic ethanol

D

Serum ALT
Inflammasome signaling through extracellular vesicles

It has been reported by our group and others that LMNCs communicate through exchange of cytoplasm via extracellular vesicles, and this cargo is pro-inflammatory in ALD\textsuperscript{215-218}. Based on these studies and on our previous findings of circulating ASC in plasma from patients with AH, we hypothesized that ASC and other inflammasome components may be present in extracellular vesicles after ethanol exposure. To test this, we stimulated THP1 monocytes with PMA for 48 hours to induce a macrophage phenotype and then stimulated with LPS and Nigericin, a potent and well-studied NLRP3-inflammasome activator. We isolated extracellular vesicles from the supernatant for presence of ASC. At the same time, we used the cell pellet as positive control for the presence of ASC protein in our western blot analysis. We detected the presence of ASC protein in lysate from extracellular vesicles isolated from supernatant of stimulated cells, which was absent from non-treated cells. (Fig. 3.20) Although this data is still preliminary, it suggests an ability of cell-to-cell communication of inflammasome components via extracellular vesicles in an \textit{in vitro} system.
Figure 3.20 THP1-derived ASC in extracellular vesicles

Human monocyte cell line was stimulated with PMA to induce a macrophage phenotype (48 hours). Cells were then stimulated with LPS (10ng/mL) and nigericin to induce inflammasome activation. Supernatant and cell pellet were collected. Extracellular vesicles were purified using ExoQuick precipitation overnight and analyzed via western blot for the presence of ASC protein.
**Figure 3.21 Summary of NLRP3 inflammasome activation in ALD**

ATP and uric acid are released by ethanol-damaged hepatocytes. Kupffer cells are activated by LPS from the gut (signal 1), which license inflammasome assembly. P2X7-mediated ATP and/or uric acid (signal 2) activate the inflammasome upon recognition by NLRP3, which activates Caspase-1. Active Caspase-1 cleaves pro-IL-1β into its mature and active form, which signals through the IL-1 receptor.
**Spleen Tyrosine Kinase**

Spleen tyrosine kinase (SYK) is a regulator of signal transduction with broad reach across multiple signaling sequences. SYK is part of the Src-family and is a 72-kDa non-receptor tyrosine kinase, initially identified in hematopoietic cells\(^\text{219}\). SYK has recently been identified as a therapeutic target in hepatocytes in hepatitis C virus infection\(^\text{220}\). SYK is expressed in the cytoplasm and nucleus of hepatocytes, immune cells and other cell types\(^\text{221}\). SYK functions mainly in the transmission of inflammatory downstream signals through components of innate immunity. Specifically, SYK modulates activation of TLRs, which are among the first receptors to be activated in response to pathogen-associated molecular patterns (PAMPs)\(^\text{222}\). Upon activation, SYK regulates downstream signaling events that drive inflammatory pathways in innate immunity. Inhibition of SYK in macrophages reduced signaling through TLR4 and reduced production of IL-6, MCP-1, TNFα, and cleaved IL-1β\(^\text{222,223}\). SYK also plays an important role in hepatic neutrophils, an important pro-inflammatory cell type in alcoholic hepatitis (AH)\(^\text{92}\), through β2 integrin (CD18)-mediated extravasation of neutrophils to inflammatory lesions\(^\text{224}\). Given the broad cellular expression pattern of SYK\(^\text{219,220,225}\), as well as its diverse cellular regulatory role of signaling pathways associated with ALD\(^\text{226}\), we hypothesized that SYK may constitute a major regulator in the pathophysiology of ALD and as such, might serve as a novel therapeutic target.
Specifically, SYK regulates inflammasome activation leading to production of active IL-1β and IL-18\textsuperscript{223,227}. We found an increase in phosphorylated SYK in liver lysate and in LMNCs isolated from ethanol-fed mice\textsuperscript{228}. More importantly, mice that received daily injection of SYK inhibitor had reduced levels of Caspase-1 cleavage and secretion of IL-1β into circulation compared to vehicle treated mice\textsuperscript{164}. Together, this data suggests that SYK inhibition is a potent intervention to prevent inflammasome activation, likely through TLR4, as well as IL-1β processing in different stages of ALD, similar to its role in other inflammatory conditions.
Figure 3.22 Inhibition of SYK activation suppressed alcohol-induced inflammasome activation and IL-1β production in two murine models of ALD

WT mice were given a 10 day Lieber-DeCarli ethanol diet and sacrificed 9 hours post ethanol binge (‘Moderate ASH’); Pair-fed mice were sacrificed 9 hours post a sugar binge (A-C) or 4 weeks of Lieber-DeCarli diet (‘Chronic EtOH’). Mice received daily intraperitoneal administration of SYK inhibitor (‘SYK Inh.’) or vehicle (‘Veh.’). Total RNA was extracted from mouse livers and assessed by RT-qPCR for *IL-1b* mRNA normalized to *18S*. (A). Whole-cell liver lysate was probed for the presence of cleaved Caspase-1 protein, and quantified using ImageJ. (B). Serum samples from mice were analyzed by via ELISA for IL-1β (C,D). A P value <0.05 was considered statistically significant by analysis of variance for 6-8 mice per experimental group (*P < 0.05 compared to baseline; #P < 0.05 compared to EtOH 1 vehicle group).
**Figure 3.23 Summary of SYK signaling in ALD**

This illustration summarizes our findings on SYK signaling from various models of ALD. In the first panel, SYK modulates ERK phosphorylation and NFκB activation. In the second panel, SYK regulates inflammasome signaling by controlling TLR4 and NLRP3 activation. In the third panel, SYK controls changes in ROS- and MCP-1-dependent gene expression in hepatocytes resulting in decreased free fatty acid oxidation and increased free fatty acid synthesis. In the fourth panel, SYK regulates TBK1 and IRF3 activation through TLR4, resulting in increased Caspase-3 cleavage.
Immune cell infiltration
Inflammation
Steatosis
Hepatocyte death/Liver Injury

Alcoholic Steatohepatitis
CHAPTER FOUR: BILE ACID AGONIST SIGNALING

FXR and TGR5 signaling

*Role as transcription factor*

Bile acids (BAs) act as signaling molecules, activating two dedicated receptors: farnesoid X receptor (FXR), a member of the nuclear receptor superfamily of transcription factors, and transmembrane G protein-coupled receptor 5 (TGR5)\(^{229}\). FXR (BA receptor NR1H4) and TGR5 (also known as G-protein coupled bile acid receptor 1) regulate BA synthesis, lipid, glucose and energy homeostasis, as well as inflammation and fibrosis, all of which are important components in the pathogenesis of nonalcoholic steatohepatitis (NASH) and ASH\(^{230,231}\). Recent reports have highlighted the potential of synthetic agonists for these receptors in various liver disease models and in the treatment of different chronic liver diseases\(^{232,233}\). The FXR agonist obeticholic acid (OCA, also known as INT-747) has been recently approved for the treatment of primary biliary cholangitis (PBC), a chronic autoimmune liver disease\(^{234}\). This drug has also shown promising results in the treatment of NASH in a Phase 2 trial\(^{235}\), demonstrating anti-inflammatory and anti-fibrotic effects in the liver, and is currently in a Phase 3 clinical trial in NASH (ClinicalTrials.gov identifier, NCT02548351; sponsored by Intercept Pharmaceuticals).
Although NASH and ASH have distinct etiologies, both liver diseases have overlapping pathogenic mechanisms, including steatosis, inflammation and liver injury. Here we have tested the hypothesis that FXR and/or TGR5 agonists may be therapeutic in the treatment of early ASH in mice.

**Bile receptor agonists**

*BA receptor agonists attenuate liver injury in acute ethanol liver injury*

We found that in the binge ethanol injury model, pre-treatment with OCA and INT-777 resulted in decreased hepatic microvesicular steatosis as seen in H&E histology, while the FXR/TGR5 dual agonist, INT-767, resulted only in mild improvement of these histological features and liver injury (Fig. 4.1A,D). We then analyzed liver lipid accumulation via Oil-Red-O staining and found reduced levels in the percent of positive Oil-Red-O area (Fig. 4.1B,C) with each BA receptor agonist treatment, albeit only OCA showed a statistically significant reduction compared to vehicle pretreatment. The superior protection from steatosis by OCA after acute ethanol administration correlated positively with decreased serum ALT levels, suggesting that in this model of early ethanol-induced liver injury, OCA protects from liver damage and steatosis. In the acute binge ethanol model, mice pre-treated with the FXR agonist OCA had significant protection from liver injury, as measured by serum ALT, compared to ethanol-binged mice treated with vehicle.
Figure 4.1 Bile acid agonists attenuate liver injury and microvesicular steatosis after acute ethanol injury

WT mice treated with bile acid agonist or vehicle were exposed to ethanol or sugar binges. Liver injury and microvesicular steatosis was assessed by H&E histology on liver sections (A-C) and serum ALT (D). Steatosis was assessed by Oil-red-o staining (B), and percent-positive staining was quantified by ImageJ from the average of five random images per sample (C). P values. *P < 0.05 compared to baseline, **P < 0.001 compared to baseline, #P < 0.05 compared to vehicle-treated ethanol group. N=5 for Pair-fed, 10 for ethanol, per treatment.
BA receptor agonists attenuate liver injury and steatosis in early ALD

We similarly analyzed mouse liver sections via H&E histology and Oil-Red-O staining after prolonged ethanol administration that resulted in liver injury and steatosis compared to pair-fed mice (Fig. 4.2A,B). Mice treated with vehicle plus ethanol had substantial accumulation of macrovesicular steatosis (Fig. 4.2A), a phenotype not present in the acute liver injury model. Furthermore, H&E staining showed that mice treated with OCA, INT-767, or INT-777 had decreased hepatic macrovesicular steatosis in the liver compared to the vehicle-treated ethanol group. Likewise, Oil-Red-O staining showed a significant decrease in steatosis after treatment with each of the three BA receptor agonists (Fig. 4.2B,C).

To test if changes in serum ALT correlated with changes in liver structure, we performed liver histology. Prolonged ethanol administration resulted in liver injury indicated by elevated serum ALT (Fig. 4.2D). Interestingly, OCA treatment did not attenuate liver damage after 12 days of chronic ethanol administration. In this prolonged alcohol feeding model that causes features of early alcoholic liver disease, only treatment with the FXR/TGR5 dual agonist INT-767 reduced serum ALT levels significantly compared to the vehicle-treated ethanol group.
Figure 4.2 Bile acid agonists attenuate liver injury and macrovesicular steatosis after prolonged ethanol injury

WT mice treated with bile acid agonist or vehicle were exposed to 12 days of prolonged ethanol or pair-fed liquid diet. Liver injury and macrovesicular steatosis was assessed by H&E histology on liver sections (A-C) and serum ALT (D). Steatosis was assessed by Oil-red-o staining (B), and percent-positive staining was quantified by ImageJ from the average of five random images per sample (C). P values. *P < 0.05 compared to baseline, **P < 0.001 compared to baseline, #P < 0.05 compared to vehicle-treated ethanol group. N=5 for Pair-fed, 10 for ethanol, per treatment.
(A) Pair-Fed, EtOH

Vehicle

OCA

INT-767

INT-777

(B) Pair-Fed, EtOH

Vehicle

OCA

INT-767

INT-777

(C) Oil-Red-O

% Area

Vehicle, OCA, INT-767, INT-777

(D) Serum ALT

UL

Vehicle, OCA, INT-767, INT-777
Regulation of NLRP3

Ubiquitination of NLRP3 by PKA

We hypothesized that perhaps BA receptor agonists were inhibiting inflammasome signaling. To test this, we first probed for Caspase-1 activity in the liver. We found that there was a decrease in Casp-1 cleavage (Casp-1p10), in both acute and prolonged ethanol models, indicative of inflammasome activity (Fig. 4.3A,B). To test if the mechanism by which the inflammasome was inhibited was through the recently reported activation of protein kinase A (PKA), activated by cyclic AMP, resulting in ubiquitination of NLRP3\textsuperscript{236}. Our lab has previously shown that the NLRP3 sensor is a key driver of inflammation in alcoholic liver disease through activation from uric acid and ATP, released by damaged hepatocytes\textsuperscript{163,237}. We found an increase in phosphorylated PKA substrate, suggesting of PKA activity, especially with the TGR5 agonists (Fig. 4.3C). To test if PKA activity resulted in increased ubiquitination of NLRP3, we performed immunoprecipitation of NLRP3 and probed for ubiquitin. We found an increase in ubiquitin-tagged NLRP3, especially with INT-767, the dual agonist (Fig. 4.3D). Taken together our data here indicate that BA receptor agonists lower steatosis and improve liver pathology in ALD models by targeting NLRP3 inflammasome signaling as well as reducing steatosis in ALD.
Figure 4.3 Bile acid agonists regulate NLRP3 inflammasome via PKA

WT mice treated with bile acid agonist or vehicle were exposed to ethanol or sugar binges, or to 12 days of prolonged ethanol or pair-fed liquid diet. Effects on inflammasome activity were assessed by probing for Caspase-1 cleavage in liver lysate by western blot in the acute (A) and prolonged ethanol feeding model (B). Protein kinase A (PKA) activity in the prolonged ethanol model was assessed by probing for phospho-PKA substrate in liver lysate (C). Ubiquitination of NLRP3 was assessed by immunoprecipitation of NLRP3, followed by probing of ubiquitin protein by western blot (D).
A  Acute binge model

B  Prolonged EtOH

C

D
Regulation of steatosis through bile acid receptors

In recent years, the role of FXR in lipid and glucose metabolism has been explored\textsuperscript{238-241}. FXR activation lowers plasma and liver triglyceride by enhancing plasma triglyceride clearance\textsuperscript{242} and repressing sterol regulatory element–binding protein (SREBP) 1c-mediated hepatic lipogenesis\textsuperscript{243}. Our data in Figure 4.4A-C shows a significant reduction in FASN protein expression in the liver after treatment with FXR/TGR5 agonists. Therefore, it is likely that BA receptor agonists likely affect fatty acid synthesis pathways directly. The lipogenic and lipolytic changes correlate with previously described reversal in pathology associated with the development of ALD\textsuperscript{164,228}. 
Figure 4.4 Bile acid agonists decrease steatosis through FASN downregulation

WT mice treated with bile acid agonist or vehicle were exposed to ethanol or sugar binges, or to 12 days of prolonged ethanol or pair-fed liquid diet. Fatty acid synthase expression was assessed by probing for FASN in liver lysate by western blot in the acute (A) and prolonged ethanol feeding model (B). Individual western blots are shown in (C), where acute is shown first followed by prolonged ethanol administration, listed in groups according to treatment. Quantification was done via ImageJ.
A. **Acute Ethanol**

- **FASN**
  - Vehicle
  - OCA
  - INT-767
  - INT-777

B. **Prolonged Ethanol**

- **FASN**
  - Vehicle
  - OCA
  - INT-767
  - INT-777
Discussion

Notwithstanding significant advances in understanding the pathogenesis of ALD, very few treatment options are approved\textsuperscript{22,244,245}. Here we tested the therapeutic potential of BA receptor agonists in two mouse models of alcoholic liver injury. Our data show that in the acute liver injury model, OCA was more effective compared to the two other agonists, while in the chronic or early ALD model the dual FXR/TGR5 agonist, INT-767, shows better therapeutic efficacy compared to OCA and INT-777.

FXR and TGR5 play key roles in ALD by modulating BA metabolism, lipid and glucose metabolism, as and well as liver regeneration\textsuperscript{246}. Alcohol consumption induces hepatic lipid accumulation, immune cell infiltration and increases oxidative stress. More importantly, chronic alcohol consumption results in increased BA pool and decreased excretion of BA, suggesting that alcohol consumption may affect enterohepatic circulation\textsuperscript{247,248}. However, acute alcohol exposure has been reported to induce BA synthesis in humans and primary hepatocytes\textsuperscript{249,250}. Moreover, OCA has been shown to reduce bacterial translocation\textsuperscript{251}. Hence, we chose to test both acute and prolonged alcohol exposure models. Treatment with OCA, INT-767, or INT-777 was effective in reducing ethanol-induced steatosis after acute or chronic ethanol administration. However, only mice treated with FXR agonist OCA showed protection from acute binge ethanol-induced liver injury, whereas mice treated with the dual agonist INT-767 showed liver protection after chronic ethanol exposure. Our findings
demonstrate that treatment with FXR and/or TGR5 agonists results in improved biochemical and histological features of early ALD with varying degrees of efficacy depending on the mode of ethanol administration.

BA s repress the transcription of cytochrome P450 7A1 (CYP7A1), which catalyzes the rate-limiting step in BA biosynthesis\textsuperscript{252}. CYP7A1 is the rate-limiting enzyme involved in BA synthesis from cholesterol, and the negative feedback loop resulting in decreased bile acid synthesis may indirectly result in decreased steatosis. Goodwin et. al. showed that FXR ligand treatment induces expression of small heterodimer partner 1 (SHP-1), an atypical member of the nuclear receptor family\textsuperscript{253}. SHP-1 represses expression of CYP7A1 by inhibiting the activity of liver receptor homolog 1 (LRH-1), an orphan nuclear receptor that is known to regulate CYP7A1 expression positively. In our models, pre-treatment with OCA and INT-767, both of which signal through FXR (but not INT-777), showed increased SHP1 and decrease in CYP7A1 mRNA expression, confirming that administration of these FXR agonists has a measurable effect in the liver of mice undergoing alcohol-induced liver injury.

We assessed the effect of FXR/TGR5 agonists in protecting against the alcohol induced inflammation. Although there was no impact on TNF\alpha and MCP-1 in both models of alcoholic liver injury, the $\text{IL-1}\beta$ mRNA was consistently reduced in both models of ethanol administration. This suggests that bile acid agonists may play a role in repression of inflammasome signaling, a key driver of inflammation in
ALD\textsuperscript{237}. This finding was further supported by the recent report identifying that bile acids negatively regulate the NLRP3 inflammasome\textsuperscript{236}.

Together, our study based on two murine models of ethanol administration are supported by a report in which FXR agonist (6ECDCA) treatment resulted in decreased hepatic steatosis and oxidative stress in a different model of ethanol-induced liver injury (ethanol combined with a low-protein diet)\textsuperscript{254}. This finding by Lívero et al. suggests that reduced lipid accumulation was mediated by FXR regulation of FASN and sterol regulatory element-binding protein (SREBP1C, also referred to as Srebpf1) expression\textsuperscript{255}. Our findings here demonstrate that FXR/TGR5 activation ameliorates murine alcoholic liver disease and thus provide the first evidence of the therapeutic potential of the FRX/TGR5 agonists in treatment of alcoholic liver disease.
CHAPTER FIVE: DISCUSSION

Targeting IRF3 in disease

In the liver, IRF3 is activated by STING, an ER resident protein, in the early phase of liver injury. There are several known intracellular dsDNA sensors that activate STING signaling in response to viral infection. The cyclic dinucleotide 2’3’-cGAMP, synthesized by the dsDNA cytosolic sensor cGAMP synthase (cGAS), activates STING. We identified cGAS as key activator of IRF3 in the early phase of liver injury from ethanol exposure. We specifically identified the role of cGAS most prevalent during the acute phase of an acute-on-chronic liver injury. Interestingly, cGAS-KO mice had comparable liver injury to WT during ten days of chronic ethanol administration, suggesting that the involvement of cGAS and STING is predominantly during the early phase of liver injury, after which other overlapping IRF3 activators, such as TLR4, may play a more important role. To test if STING and IRF3 activation was dependent on mitochondrial (mt) DNA released into the cytoplasm and circulation, we measured mtDNA in plasma. Ethanol binge in mice results in increase in circulating mtDNA at two and four after the binge. Human cirrhotic livers had increased oxidative DNA damage, as measured by 8-OHdG immunohistochemistry. Further testing by immunofluorescence staining revealed 8-OHdG staining to be cytoplasmic and not nuclear. To test if mitochondrial DNA was responsible for ethanol-induced IRF3 activation, we depleted Huh7 cells, a
human hepatoma cell line, of mtDNA by incubation with ethidium bromide for four weeks. We found that mtDNA-depleted cells had no IRF3 phosphorylation from ethanol in vitro, compared to WT Huh7 cells.

Previous studies described the role of STING activation in viral infections in immune cells. In hepatocytes, we demonstrated earlier that STING and IRF3 mediate the pathogenesis in alcoholic liver disease, a role independent of microbial components. In the present study, our data demonstrate a hepatocyte-specific role of STING and IRF3 in mediating hepatic apoptosis as an early event in the development of liver fibrosis.

Hepatocytes are responsible for metabolism and for detoxifying portal blood, which can be rich in bacterial products. For this reason, innate immunity plays an important role in the liver; even though this organ is rich in immune cells, parenchymal cells have an abundance of receptors and are capable of initiating immune signaling. In our study, TBK1-mediated ER stress was detected early after ethanol or CCl4-administration only in hepatocytes and not in LMNCs.

Regulated IRE1-dependent decay of mRNA (RIDD), a key component of the unfolded protein response, results in degradation of viral mRNA transcripts and spliced Xbp1. However, there are multiple lines of evidence suggesting that IRF3 activation can result from other forms of noninfectious cell damage that involve Xbp1 splicing. Here we show that STING activation may provide the link between induction of ER stress and IRF3. Moreover, the rapid nature of the phosphorylation of IRF3 and Xbp1 splicing in our experiments due to an
acute CCl₄-induced liver injury underscores the importance of STING and its
location in the ER. Altogether, activation of STING and IRF3 may be targeted
therapeutically via protein folding chaperone or ER stress inhibitors, such as 4-
PBA or TUDCA. Our novel in vivo data demonstrate a potential for attenuating
liver injury and associated liver inflammation through attenuation of ER stress. It
would be beneficial to assess if other forms of liver injury similarly involve ER
stress and subsequent activation of IRF3. We tested a separate model of fibrosis
using the carbon tetrachloride model and found a similar IRF3- and STING-
driven phenotype, but multiple varies of approaches would be better. For
example, given the similarities between NASH and ASH, it is possible that cGAS
and STING play at least a partial role in fatty liver disease. It would be important
to assess this carefully, since the subtle and short-lived signal of IRF3
phosphorylation could easily lead to false-negative results and incorrect
interpretation of the data.

Viral infections as well as noninfectious injuries are associated with
induction of cell death signals, emphasizing the importance of the BH3-domain
within IRF3 as responsible for its pro-apoptotic properties in the mitochondria. It
is also possible that ethanol or CCl₄-induced cell death signaling from STING
activation of IRF3 and BAX may trigger ER membrane permeability²⁶⁵ in addition
to the effects on the mitochondrial membrane integrity.

Pro-apoptotic signaling may play a beneficial role in the resolution of
inflammation after liver injury, specifically when it is targeted to infiltrating
immune cells such as neutrophils. Uncontrolled parenchymal apoptosis, however, although typically controlled and tightly regulated, may be the tipping point responsible for subsequent necrosis. Our data suggest that overwhelming levels of IRF3-mediated apoptosis may eventually lead to a form of secondary necrosis. Taken together, our data provides strong evidence that inhibition of parenchymal apoptotic signaling is sufficient to reduce necrosis and amplified liver damage.

In conclusion, our study builds on the previous paradigm of ALD. The liver damage, ER stress and death of hepatocytes is mediated by the engagement of the ER adaptor protein, STING, with phosphorylated IRF3, resulting in activation of the mitochondrial pathway of hepatocyte death. As such, fibrosis observed in ALD is more than a pathology strictly driven by inflammation. Rather, hepatocyte death appears to be a separate key component in the process of liver fibrosis. In addition, as summarized in Figure 3.23, propagation of cGAS-mediated cyclic dinucleotides via hepatic gap junctions allows for amplification of liver injury. This was abrogated with use of the small-molecule inhibitor 2-APB.

For future direction, I hypothesize that it is perhaps not IRF3 phosphorylation but rather merely its association with STING and TBK1 that license its pathogenic effect. To test if this is mediated by its BH3-domain or through its phosphorylation, I propose creating a IRF3-mutated mouse in which its BH3 motif has been mutated, but its phosphorylation sites remain intact. The
use of such mice would help elucidate the contribution of its association with Bax from its phosphorylation and activation.

**Targeting Inflammasome activators**

Ethanol cause release of damage-associated molecular patterns (DAMPs) from damaged cells. We studied if hepatocyte-derived DAMPs were ligands needed for inflammasome activation. Our lab previously showed that ethanol exposure results in inflammasome activation and maturation of interleukin (IL)-1β, a key pro-inflammatory cytokine in ALD. Oligomerization of the adaptor protein ASC (Apoptosis-associated speck-like protein containing CARD) is essential for inflammasome activation. Upon inflammasome activation, Caspase-1 is activated which leads to cleavage of pro-IL-1β into active IL-1β. IL-1β has been shown to be required for the development of alcohol-induced liver inflammation. Inhibition of IL-1 signaling with Anakinra, an IL-1R antagonist, is therapeutic in a mouse model of ALD. Extracellular ASC ‘specks’ that retain bioactivity in vitro are released during pyroptosis, a caspase-1 mediated cell death. However, the role of ethanol-induced pyroptosis and extracellular ASC has not been identified in ALD.

We found that ATP and uric acid are two essential DAMPs released by damaged hepatocytes, and not liver mononuclear cells, upon exposure to ethanol. These DAMPs activate the inflammasome through NLRP3 sensor.
Using various genetic knockout and transgenic mice, we identified that uric acid and ATP signaling are needed for inflammasome signaling in an in vivo murine model of ALD. Moreover, therapeutic intervention in mice with allopurinol or probenecid, two FDA-approved compounds that promote secretion of uric acid, are effective in treating inflammation and liver injury associated with ALD. When dissecting the early events associated with liver injury and inflammation, we found that inflammasome signaling further augments hepatocyte injury and DAMP release in vivo, in a feed-forward loop. Interestingly, inhibition of IL-1 receptor, using Anakinra, enhances hepatocyte regeneration and recovery from ethanol-induced liver injury. We have also identified MCC950 as a viable therapeutic approach in targeting NLRP3 activation in vivo. The use of MCC950 poses the potential benefit over Anakinra at preventing pyroptosis and subsequent gasdermin D cleavage. This is especially important given our new findings of extracellular ASC, in circulation and as liver aggregates.

We analyzed plasma of healthy volunteers and patients with acute hepatitis for the presence of ASC protein. Acute hepatitis patients had increased soluble ASC protein in the circulation, as measured by ELISA and confirmed with immunoprecipitation. On immunohistochemistry, all ten livers from patients with alcohol-induced cirrhosis but none of the controls had extensive ASC aggregates in extracellular spaces. Using immunofluorescence, we observed extracellular ASC aggregates and ASC oligomers with immunoprecipitation in mouse livers with acute hepatitis. We hypothesized that extracellular ASC was dependent on
liver macrophages undergoing pyroptosis, a necrotic form of inflammasome-dependent programmed cell death. We found increased ethanol-induced pyroptosis and apoptosis indicated by a decrease in AnnexinV-positive macrophages from mice pre-treated with Caspase-1 or pan-Caspase inhibitors, respectively. Pre-treatment with a necroptosis inhibitor had no effect on cell death. Moreover, pre-treating mice with MCC950, an NLRP3 inhibitor, reduced levels of Caspase-1 activity, macrophage pyroptosis and steatohepatitis in acute hepatitis in mice. Finally, we detected an increase of cleaved Gasdermin D, the trigger needed for Caspase-1-mediated pyroptosis, in human cirrhotic livers and in mouse livers with alcoholic hepatitis.

A recent study by our group focused on targeting upstream activators of the inflammasome by daily administration of allopurinol or probenecid (100 mg/kg), two FDA-approved medications for the treatment of hyperuricaemia in patients with gout that decrease urate through differing pathways\textsuperscript{152}. Allopurinol is a free radical scavenger and an inhibitor of xanthine oxidase\textsuperscript{267}, an enzyme required for uric acid synthesis. Probenecid promotes renal excretion of uric acid. Both therapies effectively lowered serum and liver uric acid levels, as well as protected from ethanol-induced inflammation, liver injury and steatosis in a mouse model of chronic alcoholic steatohepatitis\textsuperscript{237}.

Our findings of allopurinol administration being protective from ethanol-mediated liver injury in mice is supported by a previous study by Kono H., et al. in 2000 using the Tsukamoto and French model in rats\textsuperscript{153}. Interestingly, our results
indicate that probenecid provided superior protection from ethanol compared to allopurinol. This is likely due to probenecid’s inhibition of ATP signaling via pannexin-1 channels\textsuperscript{154}, on top of its promotion of uric acid secretion, thus providing a two-pronged approach targeting upstream activators of the inflammasome.

In addition, recombinant uricases have been used in the treatment of gout\textsuperscript{152}. Interestingly, in a genetic model mimicking recombinant uricase therapy, our group found attenuation of inflammation and liver injury from chronic ethanol administration in transgenic mice overexpressing two forms of uricase\textsuperscript{237}. As such, it is plausible that recombinant uricase, in combination with other drugs, may provide as a viable therapy for treatment of alcoholic steatohepatitis.

Increasing evidence suggests that cumulative danger signals contribute to inflammasome activation in NASH. Gut-derived microbial danger signals, such as LPS and/or bacterial DNA, are likely to provide inflammatory signals. In addition, as a result of hepatocyte damage, there are several sterile inflammatory signals in NAFL and NASH to provide the second signal for inflammasome activation. These sterile signals include but are not limited to fatty acids, uric acid, ATP, HMGB1 and likely other factors\textsuperscript{268}. For this reason, it is especially important be mindful of the potential contribution of the microbiome to the inflammatory microenvironment in the liver. Early on, we established a standard operating procedure of exchanging bedding of mice between groups to help minimize contributions from microbiota. We also ensured that all of our WT mice, which
were purchased through Jackson Laboratories, would have at least four to five days to acclimate in our mouse facility. This would help their microbiome reach equilibrium with our genotype-specific mouse colony that we tested. We used littermate control mice for our transgenic mouse studies, which is considered best practice. The decision was made that having gender and age-matched controls served as a higher priority than having littermate controls. Due to limitations on space within our animal facility and prohibitive costs associated with rearing our own WT controls, it was not possible for all other mouse experiments to use have littermate, age- and gender-matched controls.

On the topic of inflammation, IL-1β, after engaging the IL-1 receptor (IL-1R), amplifies pro-inflammatory cytokine production including IL-1β, TNFα, MCP-1, and IL-6. In addition, IL-1β propagates effects that drive to pathological features associated with alcoholic liver disease. Specifically, IL-1β promotes steatosis, hepatocyte death, liver fibrosis and inhibits hepatocyte regeneration. All of these biological effects of IL-1β and IL-1R activation are blocked by the naturally occurring, endogenous IL-1 receptor antagonist (IL-1ra). Anakinra is a recombinant version of IL-1RA that blocks the effects of IL-1β by inhibiting the binding of IL-1β to IL-1R, was FDA-approved for treatment of moderate to severe rheumatoid arthritis (RA) in 2001, and is used also in patients with Still's disease among other auto-inflammatory diseases. However, given the potential role of post-pyroptotic signaling, the efficacy of MCC950
compared directly with Anakinra is an important future step to undertake to prevent NLRP3 activation, upstream of IL-1R.

For future direction, it is important to assess in more detail the role of ACS specks. For this, I would recommend the use of a highly inflammatory model such as the 4-week plus three-binge model, where plasma is isolated at various time-points after the final binge. The best mice to use for these experiments would be ASC-GFP transgenic mice developed by Dr. Doug Golenbock. Visualizing ASC speck formation from Kupffer Cells and macrophages isolated from liver and circulation, respectively, using the Imagining Flow Cytometry technology from Amnis FlowSight would be the perfect tool for these experiments. It would be critical to assess the role of Gasdermin D in this model, and directly compare the role of Anakinra in parallel to MCC950 or VX-765 in attenuating Gasdermin D cleavage. I hypothesize that MCC950 and VX-765 would both provide superior protection from Gasdermin D cleavage and ASC speck formation than Anakinra due to their ability to inhibit inflammasome activation rather than its mere amplification.

**Potential for role of bacterial dinucleotides**

I hypothesize that bacterial messenger molecules may activate IRF3 early after ethanol administration. Based on our previous findings that serum endotoxin levels increase early, peaking between one to two hours, and that phosphorylation of IRF3 peaks between two and four hours, along with the
detection of 16S bacterial DNA in human serum after ethanol consumption, we hypothesize that c-di-GMP, cyclic dinucleotides synthesized by bacteria may pass through the lining of the gut along with LPS. This might explain the progression of early phosphorylation of IRF3 in hepatocytes concurrent with endotoxin infiltration into systemic circulation and the liver. Ultimately these events are followed by hepatocyte death and liver inflammation, indicated by increased ALT and TNFα, IL-1β, and MCP1, peaking at eight hours. For this reason, I hypothesize the detection of cyclic dinucleotides using mass spectrometry, both mammalian and bacterial in circulation in vivo of both humans with severe ALD and mice after acute-on-chronic ALD. Furthermore, I propose administration of diguanylate cyclase inhibitors in vivo to inhibit c-di-GMP synthesis and secretion by intestinal bacteria after ethanol administration to determine if bacterial diguanylate cyclase is a viable therapeutic target to treat ethanol-induced liver injury. Finally, to visually assess the role of cyclic dinucleotides in relation to ethanol-induced zonal distribution of liver injury, creating an AAV-mediated biosensor for detection of cyclic dinucleotide binding would be very informative. To this end, I hypothesize that creating biosensors onto an AAV8-mediated in vivo riboswitch-lacZ reporter gene of cyclic dinucleotide signaling by injecting mice with an AAV8 vector encoding β-galactosidase under control of a riboswitch-heptamere lacZ construct, so that cells responding to cyclic dinucleotide signaling are labeled by β-galactosidase expression.
Lastly, as a therapeutic approach, if administration of diguanylate cyclase inhibitors is not effective or somehow has deleterious side-effects as part of the ethanol liquid diet, I propose an alternate approach to therapeutically target bacterial cyclic dinucleotides after they have crossed the intestinal wall via hepatic overexpression of PdeB, a c-di-GMP specific phosphodiesterase, using recombinant AAV8-mediated gene therapy. I hypothesize that ectopic expression of PdeB transgene via rAAV8 will drive hydrolysis of intracellular c-di-GMP, thereby reducing activation of STING and subsequent phosphorylation of IRF3.
MATERIALS AND METHODS

Animal studies. All WT mice were obtained from Jackson Laboratory, Bar Harbor, ME as 6- to 8-week-old female C57Bl/6 WT. Littermate controls, bred at in our lab, were used for the uricase transgenic mouse experiments. IRF3-deficient (IRF3-KO) or type I IFN α/β receptor 1-(IFNAR1)-KO mice (provided by J. Sprent, Scripps Research Institute, La Jolla, CA, USA), Casp-1/11-KO (gift of A. Hise, Case Western Reserve University, Cleveland, Ohio, USA) and IRF7-KO mice (provided by T. Taniguchi, Tokyo, Japan), all on C57Bl/6 background, were used. The STING-deficient Tmem173gt (goldenticket) mice (provided by R. Vance, University of California, Berkeley, CA, USA,) and the TRAM-KO mice (provided by S. Akira, Osaka University, Japan) were on the B6.129sf2 background; we used B6.129sf2 WT mice (Jackson Laboratory) as controls for these two strains. Please refer to Table 2 for a list of mouse genotypes, genetic background and sourcing information.

Ethanol administration. WT C57BL/6 6- to 8-weeks-old female mice were purchased from Jackson Laboratories and were cohoused in the University of Massachusetts Medical School Animal Medicine Facility for one week prior to the start of the experiment at which time they were doubly housed. Some animals were fed with the Lieber DeCarli ad libitum diet (chronic model) or received intragastric ethanol (5g/kg body weight, acute model). Mice were treated with an acute-on-chronic alcohol feeding model, as described by Bertola et al. Briefly,
all mice were fed the Lieber DeCarli pair-fed diet for five days to become acclimated to a liquid diet. Some mice were then switched to the Lieber DeCarli ethanol diet containing 5% ethanol and maltose dextran (to control for caloric intake). Pair-fed mice were calorie matched with the ethanol-fed mice. On the tenth day, mice were gavaged between midnight and 2am with either ethanol (5 g kg\(^{-1}\) body weight) or iso-caloric maltose dextran. Mice were cheek bled then euthanized 9h post-gavage (9-11am), unless indicated otherwise. For assessment of peak phosphorylation of IRF3 or cleavage of inflammasome-related proteins, the time-point of four-hours post binge ethanol was used. All animals in this study were cared for in accordance with the Institutional Animal Care and Use Committee regulations at the University of Massachusetts Medical School.

In vivo treatment of mice. Some animals received a single dose of 25 mg/kg recombinant IL-1 receptor antagonist (IL-1Ra, Anakinra; Amgen). Anti-CD95 antibody, clone: Jo2, 0.5 mg/kg (BD biosciences, San Jose, CA, USA), or saline was administered as a single i.p. injection. All animals received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School.

Kupffer-cell depletion. WT mice were injected with 200 μL liposomal clodronate or liposomal PBS (purchased from N. van Rooijen, Free University Amsterdam,
Amsterdam, the Netherlands) via tail vein injection. 48 hours later, mice were sacrificed to evaluate KC depletion using F4/80 staining (Cl:A3-1, AbD Serotec, Raleigh, NC, USA), or administered a single intragastric dose of ethanol.

*Primary hepatocyte isolation.* Isolation of primary mouse hepatocytes and liver mononuclear cells was performed as follows: Mouse livers were perfused through the portal vein on anesthetized mice with Buffer 1 (0.5mM EGTA in HBSS) for 5 minutes, followed by Buffer 2 (25mg Collagenase + 0.5mM CaCl$_2$ in HBSS per mouse) for 5 minutes, all pre-warmed to 37 degrees. Collagenase was reconstituted at a concentration of 100 mg/mL in HBSS + CaCl$_2$ and aliquoted into 250 µL aliquots, with 25 mg of collagenase to be used per mouse liver to be perfused. Hepatocytes were separated from dissociated livers by spinning for 5 min at 300g at 4 degrees. Cell pellet was washed three times with 2%FBS in PBS.

*In vitro experiments.* Primary hepatocytes were cultured in Waymouth’s medium supplemented with 10% fetal bovine serum and 1% insulin, transferrin, selenium solution. Primary hepatocytes were seeded in 6-well collagen-coated plates (Biocoat, Becton Dickinson, Bedford, MA). Before starting stimulation experiments, hepatocytes were rested for 4 hours. Subsequently culture media was replaced, and stimulation was performed as indicated in the figure legends. The Jo2 antibody was purchased from BD biosciences (San Jose, CA, USA),
BX795, polyI:C and lipofectamine from Invitrogen (Life Technologies Corp., Grand Island, NY, USA), and thapsigargin (1μM) from Sigma (St. Louis, MO, USA). For mitochondrial DNA depletion in Huh7 cells, we incubated cells with ethidium bromide (0.2 µg/ml) and supplemented with 50 µg/ml uridine and 1 mM sodium pyruvate for 40 days to deplete mtDNA\textsuperscript{132}. mtDNA depletion was confirmed by Cytochrome C Oxidase I (COX1) and NADH Dehydrogenase Subunit 6 (ND6) mRNA using Taqman probes (ThermoFisher Scientific).

**Biochemical assays.** Serum ALT was determined using a kinetic method (D-Tek LLC., Bensalem, PA). Colorimetric assays were used to measure liver triglycerides (Wako Chemicals, VA, USA) and the LDH activity in cell culture supernatants (Abcam, Cambridge, MA, USA). ATP in serum or cell-free supernatant was measured using Cell-Titer Glo. A standard curve was made every time with freshly reconstituted ATP in water for accurate assessment of concentrations. Uric acid in serum or cell-free supernatant was measured using colorimetric assay at first. This process was later improved in sensitivity using fluorimetric measurements after 30min incubation at 37 degrees.

**Cytokine measurement.** Levels of MCP-1 and TNF-α were measured using specific anti-mouse ELISA from Biolegend (San Diego, CA, USA). IFN-β was measured using specific anti-mouse ELISA from PBL (Piscataway, NJ, USA) and
cytochrome c and serum FasL was measured by ELISA kit from R&D (R&D systems, Inc., Minneapolis, MN).

**Protein quantification.** Liver whole-cell lysates and nuclear preparations were extracted using RIPA buffer supplemented with protease and phosphatase inhibitor cocktails. Tissue and cells were homogenized using Qiagen's TissueLyzer system, left on ice for 10 min, then spun at 10,000 RPM for 10 min, followed by removal of pelleted cell-debris. Lysates were quantified using Bradford assay or BCA assay. Lysates were then prepared using Laemllli loading buffer and boiled for 5 min. Mitochondrial and total ER fractions were prepared using specific extraction kits from Imgenex (San Diego, CA) as per manufacturer's protocol. Antibodies for western blots and their associated dilutions are described in Table 3. Antibodies for loading controls were the following: total IRF3 (phospho-IRF3), GRP78 and KDEL (ER fraction), Beta-actin, beta-tubulin (whole-cell lysate), porin (mitochondrial fraction) and TATA-binding protein (TBP) (nuclear fraction). The TrueBlot system from eBioscience (San Diego, CA, USA) was used for immunoprecipitation assays and performed as per manufacturer's instructions.

**RNA Analysis.** Cells or tissue as stored in Qiazol at 4 degrees overnight. RNA was extracted from liver tissue or cells using RNeasy (Qiagen) according to the manufacturer’s instructions, including on-column DNase digestion (Zymo
Research). cDNA was written from 1μg of RNA and then diluted 1:5 in nuclease-free water. SYBR Green (BioRad) real-time qPCR was performed according to the manufacturer’s instructions. The PCR primers used for RNA analysis are described in Table 1. For RT-PCR, 18S was used as a housekeeping gene for 2-ddCt method of RNA expression analysis. For analysis of sXbp1 mRNA, PCR was performed and products were separated on 3% agarose gel.

**Histopathological analysis.** Liver sections were stained with H&E, Sirius Red or Oil-red-O and analyzed by microscopy. Immunohistochemistry staining for F4/80 (Cl:A3-1, AbD Serotec, Raleigh, NC, USA) or TUNEL staining (Roche, Indianapolis, IN, USA), were performed in formalin-fixed, paraffin-embedded livers. OCT-embedded frozen liver sections were used for Oil-red-O staining, and quantification was performed using ImageJ to assess hepatic steatosis.

Formalin-fixed paraffin-embedded liver sections were stained with anti-mouse myeloperoxidase (MPO) antibody, F4/80 CyclinD1 and Ki67, and subsequently labeled with streptavidin-biotin immunoenzymatic antigen for detection with 3,3'-diaminobenzidine (DAB) (UltraVision Mouse Tissue Detection System Anti-Mouse HRP/DAB; Lab Vision). ImageJ (NIH) was used for image analysis and quantification.

**Statistical Analysis.** Statistical significance was determined using two-sided t-test; ANOVA and Dunnett’s multiple comparison post-test were used to compare the
means of multiple groups. Two-way ANOVA was used to determine the global effect of genotype on serum ALT. Mantel-Cox regression was used to assess statistical differences plotted in Kaplan Meier Survival analyses. Data are shown as mean ± SEM and were considered statistically significant at P < 0.05. We used Microsoft Excel, SPSS 19.0 (IBM SPSS, Chicago, IL, USA) and GraphPad Prism 7 (San Diego, CA, USA) for calculations.
### Table 1. Table of Primers

The qPCR primers used below were used for mouse gene targets.

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<th>Gene</th>
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<th>Reverse Primer</th>
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<td>18s</td>
<td>GTAACCCGTTGAACCCCAT</td>
<td>CCATCCAATCGGTAGTAGCG</td>
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<td>Tnfa</td>
<td>CACCACCATCAAGGACTCAA</td>
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<td>Mcp1</td>
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<td>Pro-II1b</td>
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Table 2. Table of Mouse Genotypes

The mouse genotypes indicated below were used in these studies. Unless indicated, all mice used were 6- to 8-week-old female C57Bl/6 WT obtained from Jackson Laboratory, Bar Harbor, ME.

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<tr>
<th>Genotype</th>
<th>Background</th>
<th>Source</th>
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<tr>
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<td>IFNAR1-KO</td>
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<td>IRF7-KO</td>
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<td>T. Taniguchi, Tokyo, Japan</td>
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<td>Tmem173&lt;sup&gt;1&lt;/sup&gt;</td>
<td>B6.129sf2</td>
<td>R. Vance, University of California, Berkeley, CA,</td>
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<td>TRAM-KO</td>
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<td>S. Akira, Osaka University, Japan</td>
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<td>ssUOX-Tg</td>
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<td>K. Rock, UMMS</td>
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Table 3. Table of Antibodies

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