THE ROLE OF CASPASE-1 IN LIVER AND ADIPOSE TISSUE DURING METABOLIC DYSREGULATION IN MOUSE MODELS OF NASH

By

LAURA JUDITH DIXON

Submitted in partial fulfillment of the requirements
For the degree of Doctor of Philosophy

Thesis Advisors: Dr. Ariel E. Feldstein M.D. and Dr. Laura E. Nagy Ph.D.

Department of Molecular Medicine
CASE WESTERN RESERVE UNIVERSITY

January, 2013
We hereby approve the dissertation of

Laura Judith Dixon

Candidate for the Ph.D. degree *.

(signed) George R. Dubyak

(chair of committee)

Laura E. Nagy

(thesis advisor)

Nizar Zein

(clinical mentor)

Alex Almasan

Amy G. Hise

(date) December 07, 2012

* We also certify that written approval has been obtained for any proprietary material contained therein.
I would like to dedicate this work to my husband Joel. Thank you for your friendship, love, and constant support, which without this work would not be possible.
## TABLE OF CONTENTS

| LIST OF TABLES | 6 |
| LIST OF FIGURES | 7 |
| ACKNOWLEDGEMENTS | 8 |
| LIST OF ABBREVIATIONS | 9 |
| ABSTRACT | 11 |
| CHAPTER 1. Introduction | 13 |
| I. Biomedical importance of NASH | 13 |
| II. Nonhepatic pathogenesis of NASH | 16 |
| III. Hepatic pathogenesis of NASH | 19 |
| The Liver | |
| Metabolism and hepatocytes | |
| Contributions of Non-parenchymal Cells to NASH | |
| Kupffer Cell and Phenotypes | |
| Liver Sinusoidal Endothelial Cell | |
| Hepatic Stellate Cell and Fibrogenesis | |
| IV. Interleukin-1, Caspase-1, and Inflammasome | 33 |
| Interleukin-1 | |
| Caspase-1 | |
| Caspase-1, the protein | |
| Regulation | |
| The Inflammasome complex | |
| Hepatic Inflammasome | |
| V. Mouse models of NASH | 39 |
| CHAPTER 2. General Methods | 42 |
| CHAPTER 3. Caspase-1 -mediated regulation of diet-induced steatohepatitis | 49 |
| Summary | |
| Introduction | |
| Results | |
| Discussion | |
| CHAPTER 4. High fat diet Induces Caspase-1 -Dependent Markers of Early Fibrosis in Mice | 73 |
| Summary | |
| Introduction | |
| Results | |
| Discussion | |
| CHAPTER 5. Discussion and Future Directions | 97 |
| I. What are the mechanisms responsible for activating caspase-1 in NASH? | 98 |
| Gut-Liver Axis | |
| The role of NASH models | |
| II. What specific cellular and molecular effects does caspase-1 have in NASH injury? | 103 |
| Hepatocytes | |
Kupffer Cells

HSC

III. Clinical Correlate 125
IV. Conclusion 127

BIBLIOGRAPHY 128
<table>
<thead>
<tr>
<th>Table 1</th>
<th>Sources of ROS</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2</td>
<td>Absence of caspase-1 protects from high fat-induced obesity</td>
<td>80</td>
</tr>
<tr>
<td>Table 3</td>
<td>Histopathological analysis of mice on the high fat diet</td>
<td>84</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.1</td>
<td>Insulin Signaling</td>
<td>18</td>
</tr>
<tr>
<td>1.2</td>
<td>Hepatic Architecture</td>
<td>20</td>
</tr>
<tr>
<td>1.3</td>
<td>HSC activation phenotypes</td>
<td>32</td>
</tr>
<tr>
<td>1.4</td>
<td>Mechanisms of Inflammasome Assembly and Caspase-1 Activation</td>
<td>38</td>
</tr>
<tr>
<td>2.0</td>
<td>Hepatic caspase-1 activation during diet-induced steatohepatitis</td>
<td>54</td>
</tr>
<tr>
<td>3.0</td>
<td>Caspase-1 suppression is associated with dissociation between hepatic triglyceride accumulation and inflammatory activity in diet-induced steatohepatitis</td>
<td>58</td>
</tr>
<tr>
<td>4.0</td>
<td>HSC activation and collagen deposition during diet-induced steatohepatitis</td>
<td>62</td>
</tr>
<tr>
<td>5.0</td>
<td>Protection from MCD-diet induced fibrosis in Casp1−/− is independent of caspase 3 activation and hepatocellular apoptosis</td>
<td>64</td>
</tr>
<tr>
<td>6.0</td>
<td>Kupffer cell depletion abrogates caspase-1 –mediated diet-induced steatohepatitis</td>
<td>66</td>
</tr>
<tr>
<td>7.0</td>
<td>Proposed model for role of inflammasome and caspase-1 activation in tissue damage and fibrosis in steatohepatitis</td>
<td>68</td>
</tr>
<tr>
<td>8.0</td>
<td>Caspase-1 and IL-1β expression is increased after high fat feeding</td>
<td>78</td>
</tr>
<tr>
<td>9.0</td>
<td>Adiposity is increased in caspase-1 knockout mice</td>
<td>81</td>
</tr>
<tr>
<td>10.0</td>
<td>Caspase-1 knockout mice are protected from high fat-induced hepatic steatosis</td>
<td>83</td>
</tr>
<tr>
<td>11.0</td>
<td>High fat-induced expression of lipogenesis-related genes is attenuated in caspase-1 knockout mice</td>
<td>86</td>
</tr>
<tr>
<td>12.0</td>
<td>High fat-induced expression of inflammatory cytokines and chemokines is attenuated in caspase-1 knockout mice</td>
<td>88</td>
</tr>
<tr>
<td>13.0</td>
<td>High fat-induced early fibrogenesis is prevented in caspase-1 knockout mice</td>
<td>90</td>
</tr>
<tr>
<td>14.0</td>
<td>Caspase-1 knockout mice are not protected from carbon tetrachloride –induced fibrogenesis</td>
<td>101</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

I would like to thank my two mentors, Ariel and Laura for all they have done for me. Ariel’s enthusiasm for research, along with his passion for patients has inspired me. I thank Laura for taking me on as a third-year student with two projects. She has truly taught me much about science and has brought me to a higher level of research. I would also like to thank the former Feldstein lab and the Nagy lab for supporting me throughout this journey and for making a fun work environment. Special thanks goes to Michael Berk, Samjhana Thapaliya, Megan McMullen and Dola Das for experimental assistance. I would also like to thank my former and current thesis committee members for their advice. With much gratitude, I thank my parents for unending love and trust. Finally, I would thank my husband Joel for everything he is and does for me.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>αSMA</td>
<td>Alpha Smooth Muscle Actin</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine Aminotransferase</td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis Speck-like Protein containing a CARD</td>
</tr>
<tr>
<td>AST</td>
<td>Aspartate Aminotransferase</td>
</tr>
<tr>
<td>CASP1</td>
<td>Caspase-1</td>
</tr>
<tr>
<td>CLOD</td>
<td>Clodronate</td>
</tr>
<tr>
<td>COL1</td>
<td>Collagen Type I</td>
</tr>
<tr>
<td>CRP2</td>
<td>Cysteine and Glycine-Rich Protein 2</td>
</tr>
<tr>
<td>CTL</td>
<td>Control</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger Associated Molecular Pattern</td>
</tr>
<tr>
<td>Assay</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Glyceraldehyde 3-Phosphate Dehydrogenase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>HFAT</td>
<td>High Fat</td>
</tr>
<tr>
<td>HSC</td>
<td>Hepatic Stellate Cell</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin-1</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-Alcoholic Fatty Liver Disease</td>
</tr>
<tr>
<td>NAS</td>
<td>NAFLD Activity Score</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-Alcoholic Steatohepatitis</td>
</tr>
<tr>
<td>NLRP</td>
<td>Nod-Like Receptor Protein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCD</td>
<td>Methionine-Choline Deficient Diet</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Recruitment Protein-1</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil Red O</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome Proliferator Activating Protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Scd1</td>
<td>Stearoyl-CoA Desaturase 1</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol Regulatory Element Binding Protein</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor Necrosis Factor-alpha</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling</td>
</tr>
<tr>
<td>-------------</td>
<td>------------------------------------------------------------</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
The Role of Caspase-1 in Liver and Adipose Tissue During Metabolic Dysregulation in Mouse Models of NASH

Abstract

By

LAURA JUDITH DIXON

Consumption of high-energy diets and weight gain are associated with development of several metabolic complications such as insulin resistance and hepatic steatosis, a stage within the spectrum of Non-Alcoholic Fatty Liver Disease (NALFD). NAFLD is a common form of chronic liver disease that affects both adults and children. 10-20% of adults have hepatic steatosis, which is characterized by triglyceride (TG) accumulation and typically follows a benign non-progressive clinical course. Nonalcoholic steatohepatitis (NASH) is defined as lipid accumulation and cellular damage, inflammation and early to modest fibrosis. The progression of NAFLD to NASH remains incompletely understood. Therefore, dissection of mechanisms responsible for progressing this disease is an important area of investigation. In this thesis, the role of caspase-1, a pro-inflammatory protease, is explored in murine models of diet-induced NASH. Caspase-1 expression and activation is increased after both MCD and high fat diets, and was detected in hepatocyte and non-parenchymal cells. However, after feeding mice the MCD diet, fractionation of the liver further demonstrated an increase of caspase-1 and interleukin-1β only in the non-parenchymal fraction.
Casp1−/− mice on the MCD diet were protected from diet-induced increases in inflammatory markers, such as TNFα and F4/80, and increases in HSC activation marker, αSMA and the deposition of collagen-1. These effects were independent of ALT levels, apoptosis and caspase-3 expression. Deletion of macrophages in the liver by clodronate liposomes suppressed caspase-1 activation and demonstrated a protection from MCD-induced inflammation and fibrogenesis, suggesting that caspase-1 in Kupffer cells plays an important role in NASH pathogenesis. Casp1−/− mice on the high fat diet gained less weight than wild type mice, but had greater adiposity. Casp1−/− mice on the high fat diet were protected from hepatic steatosis and TG accumulation, increases in TNFα and MCP-1, but were not changed for F4/80. Casp1−/− mice on the high fat diet did not have high fat diet-induced αSMA and collagen-1 deposition, suggesting a protection from early fibrogenesis. Mice deficient in caspase-1 are protected from both MCD and high fat diet-induced NASH, specifically through steatosis, inflammation, and fibrogenesis. These data point to caspase-1 as a potential target for NASH therapy.
CHAPTER 1

Introduction

Since the original identification of caspase activation and hepatocellular apoptosis was correlated with human NASH, attention was turned toward caspase-dependent cell death in NASH pathogenesis [1,2,3]. Recent studies have identified the pro-inflammatory cytokines IL-1β, IL-1α and their receptor, IL-1R to play a role in NASH [4,5]. Therefore, its activating enzyme, caspase-1 is an attractive target for NASH therapy. In this thesis, the role of caspase-1 is investigated in two mouse models of diet-induced NASH. The expression and activation of caspase-1, IL-1β are discussed as well as caspase-1 knockout mice in diet-induced NASH. In addition, caspase-1 activation and caspase-1 activation complex (the inflammasome) are investigated in hepatic stellate cell activation in vitro.

I Biomedical Importance of NASH

The Center for Disease Control has estimated that the prevalence of NAFLD and NASH in the US is 20% and 3%, respectively. However, the prevalence of NAFLD climbs to 50% of the overweight population (BMI>25) and 25% of the obese population (BMI>30). The clinical importance of NAFLD is that if left to progress to NASH, then there is an increased risk for developing cirrhosis and hepatocellular carcinoma (HCC).
NAFLD and NASH share common symptoms. Generally, most patients have no symptoms, but may develop right upper-quadrant abdominal pain. The liver may enlarge and be inflamed, causing dull achy pain. In addition, symptoms of type II diabetes will present with NAFLD and NASH. These include diabetic retinopathy, hypertension, renal failure and cardiovascular disease. Also, a dark pigmentation of the skin may occur on the neck and armpits, which is another sign of insulin resistance. Symptoms of NASH cirrhosis include an enlarged, hardened liver. Patients also present to the clinic with visible blood vessels on the skin of the abdomen and palms of the hand. A form of cirrhosis, called decompensated cirrhosis is characterized by a small liver, swelling of the lower extremities, accumulation of fluid in the abdomen, bleeding from enlarged veins in the esophagus and mental confusion.

Recommendations for NAFLD treatment are weight loss, exercise, maintain a healthy diet, avoid alcohol, and avoid unnecessary medications. Treatment with PPAR agonists has gained recent attention for NAFLD therapy. [6]. Nagasawa et al. demonstrated that treatment of mice on the MCD diet with PPAR pan-agonist improves steatosis, inflammation and increases fatty acid oxidation [7]. In addition, drugs designed to improve insulin sensitivity are used for NAFLD therapy, including metformin and thiazolidinediones. Hepatoprotective or anti-fibrotic drugs used for NASH therapy include betaine, vitamin E, lecithin, β-carotene and selenium [8].

HCC is a major cancer that kills between 600,000 and 1,000,000 people each year. The frequency of HCC is higher in certain areas around the world, such
as Asia and Africa, but is currently increasing in Europe, Canada, and the United States [9]. The major risk factor in developing liver cancer is cirrhosis, due to a major change in the architecture of the liver, scarring and its subsequent interference with function. There is also a significant risk from HBV or HCV infection, alcohol consumption, aflatoxin B1 ingestion, hemochromatosis, or diabetes [9]. Although recent research as helped in understanding some molecular mechanisms behind HCC, diagnosed patients have little hope.

Regular 6-month surveillance by ultrasound and yearly screening for serum α-fetoprotein (AFP) of patients with known cirrhosis is the best current diagnostic care for HCC. Even so, AFP is a poor diagnostic biomarker of cirrhosis and/or HCC. It is only after surgical resection, liver biopsy, that HCC can be detected, when tumors are larger and are already at an advanced or malignant stage [10]. Patients now may present with symptoms of cirrhosis and pain, jaundice, or mental confusion. The median survival time for those with end stage cancer is three months [11]. Thus, research to discover a new diagnostic biomarker for cirrhosis and HCC is desired.

Treatments are based on the patient response to treatment, liver function, and tumor extent or size. Currently treatment options that may be curative include extremely invasive procedures such as surgery for tumor resection, liver transplantation, and the destruction of tumor tissue by ablation techniques [12]. Liver transplantation remains the most successful treatment because it removes the cancerous liver and the underlying cirrhosis. Patients with advanced HCC that is incurable or inoperable may be treated symptomatically to prolong survival.
New therapies include targeted chemotherapy such as a multikinase inhibitor called Sorafinib, which in a recent study had only a 2.8 month longer survival time than those treated with placebo [13]. Similar molecular therapies growing in popularity are angiogenesis inhibitors, telomerase inhibition, and HCC anti-antibody treatment [13].

II Nonhepatic pathogenesis of NASH

NAFLD is strongly associated with obesity and insulin resistance [14,15]. The prevalence of obesity in the United States is about 33.8% of all adults, 17% of children and adolescents [16]. There are many contributing factors in this epidemic, including lifestyle, body fat mass and distribution, and insulin resistance.

NAFLD, obesity, and insulin resistance have been correlated to lifestyle and affluence from dietary intake [17]. An increased fat mass is an important factor in NAFLD pathogenesis. Fat cells, or adipocytes are endocrine organs due to their ability to secrete proteins called adipokines, which affect surrounding cells and tissues, including liver homeostasis [18,19]. Increases in adipokines that have been correlated with NAFLD include leptin, tumor necrosis factor (TNFα), angiotensinogen, and free fatty acids (FFAs), whereas adiponectin is decreased [20]. TNF causes hepatic insulin resistance, mitochondrial permeability, decreased mitochondrial function, resulting in apoptosis [21]. TNFα activates the phosphorylation of serine residues of the insulin receptor substrate-1 (IRS-1) upon insulin binding. This serine-phosphorylated form of IRS-1 then functions to
inhibit tyrosine kinase activity of the insulin receptor (IR), which is responsible for insulin signaling [22,23,24,25]. Low levels of adiponectin in human obesity and NAFLD are indicative of insulin resistance and more severe hepatic fibrosis [20]. Not only fat mass, but also the body fat distribution contributes to NAFLD. Central adiposity, or increased visceral fat, is associated with NAFLD more so than total body fat. Therefore, increased visceral fat mass and the adipocyte-hepatocyte [26,27] crosstalk is a complex, but essential factor in NAFLD pathogenesis.

Insulin resistance has been widely associated with NAFLD [28,29]. Insulin is a hormone that is secreted from the pancreas and regulates carbohydrate and fat metabolism. Insulin facilitates liver, muscle, and fat cells glucose uptake, while decreasing glyconeogenesis and lipolysis. Insulin causes insulin receptor phosphorylation, IRS1 phosphorylation, and the activation of Phosphatidyl Inositol-3 Kinase (PI3K). This results in glucose transporter GLUT2 translocation to the plasma membrane and the uptake glucose. With insulin resistance, IRS1 phosphorylation does not take place and insulin-stimulated glucose uptake is suppressed. Extra circulating glucose then perpetuates a cycle in which the pancreas then secretes more insulin to compensate for the glucose until it become exhausted. This stage is known as type-II diabetes mellitus. Cells like adipocytes and myocytes require insulin for glucose uptake. In conditions of hyperinsulinemia, blood glucose levels rise. Hyperglycemia is a cause of a number of adverse health effects.
**Figure 1.1 Insulin Signaling.** Insulin binding to the insulin receptor causes receptor phosphorylation and subsequent IRS phosphorylation. This event triggers the activation of PI3K, which has the ability to cause GLUT translocation to the nucleus and to activate AKT/PKB. AKT then acts upon many processes, including lipolysis, fatty acid synthesis, glycogen synthesis, and protein synthesis. TNFα inhibits the phosphorylation of IRS, thus conferring insulin resistance.
III Hepatic mechanisms of NAFLD pathophysiology

The Liver

The liver is a vital organ, weighing between 1400 and 1600 grams. It is supplied by two blood supplies: 60-70% of hepatic blood comes from the portal vein, and 30-40% from the hepatic artery [30]. The portal vein and hepatic artery run in parallel, so-called portal tracts. The architecture of the liver is based on the hepatic lobule. The lobule is a hexagonal structure around the hepatic veins and the portal tracts their periphery. The hepatocytes located near the vein are called “centrilobular”, whereas those near the portal tract are called “periportal”.

Another way of classifying hepatic architecture is based on the hepatic acinus, which describes the hepatocytes as being in parenchymal zones. Hepatocytes nearest to vascular supply are classified as zone 1 and zone 3 are those furthest from blood supply, with zone 2 in between. Hepatocytes are organized into sheets with a sinusoid between (Figure 1.2). The vascular portal supply enters the sinusoids and exits via the hepatic veins, whereas arterial supply is on the other side of the hepatocyte. Hepatic sinusoids contain fenestrated epithelium.

Macrophages of the liver, Kupffer cells live on the endothelium in the lumen of the sinusoids. In between the sinusoidal epithelial cells and hepatocytes is the Space of Disse, where lie hepatic stellate cells. In addition to these main liver cells are a number of lymphocytes, T-cells, and natural killer cells and NK-T cells.
Figure 1.2 Architecture of the liver. Multiple cell types within the liver include hepatocytes, endothelial cells, hepatic stellate cells (HSC), resident macrophages (Kupffer cells). HSCs reside in the Space of Disse between hepatocytes and the sinusoidal epithelium. Upon liver injury, activated HSCs secrete extracellular matrix. Kupffer cells among other inflammatory cells reside in the sinusoid lumen. Kupffer cells become activated in response to liver injury. Delivery of inflammatory mediators and lipids through the portal circulation into the sinusoid lumen is an important causative factor in NAFLD/NASH injury.
Metabolism and hepatocytes

Originally, NAFLD disease progression to NASH was considered a two-hit process. The first hit is proposed to be insulin resistance and the accumulation of lipids within hepatocytes, considered a requirement for later events leading to liver injury. Hepatic lipid accumulation occurs from an unbalanced hepatic fat metabolism, resulting in an excess of free saturated and monounsaturated fatty acids. Contributors to part of this balance are dietary intake, hepatic uptake and hepatic synthesis.

Insulin resistance (as discussed previously) in adipocytes inhibits the action of hormone-sensitive lipase (HSL), resulting in an increase of TG hydrolysis and FFA release of adipocytes. Increased circulating FFAs increase their uptake by the liver. Another effect of insulin on hepatic steatosis comes from muscle insulin resistance. Insulin resistance in fatty myocytes increases insulin and/or glucose, consequently increasing FFA synthesis in the liver via sterol regulatory element binding protein 1-c (SREBP-1c) and peroxisome proliferators-activated receptor gamma (PPARγ). SREBP-1c transcriptionally activates genes involved in lipogenesis, resulting in increased rate of FA synthesis, contributing to steatosis. In addition, SREBP-1c activates a nuclear hormone receptor PPAR-γ, which is normally expressed at low levels in the liver. However, in the presence of insulin resistance, PPAR-γ expression is increased. The molecular mechanisms of PPAR-γ-mediated steatosis are not completely understood. However, hepatocyte specific knockout PPAR-γ mice are protected from steatosis even in the presence of insulin resistance [31].
Once in the liver, FFAs have one of two fates. They can either enter mitochondrial Beta-oxidation for degradation or be incorporated into TG for storage and export. Insulin resistance causes the activation of lipoprotein lipase, which degrades triglycerides (TG) and produces excess circulating FFAs. Excess FFAs then act as signaling molecules to nuclear receptors PPARα, thus increasing the expression of mitochondrial enzymes involved in beta-oxidation, Carnitine Palmitoyl Transferase-1 (CPT1) and medium-chain acyl-CoA dehydrogenase. The enzyme CPT1 is inhibited by malonyl-CoA, formed by acetyl-CoA carboxylase (ACC) coming from the first step in the synthesis of FA. Thus the synthesis of FA decreases the overall rate of FA oxidation, directing FAs towards the formation of TGs. However, in cases of severe steatosis, such as that of NASH, mitochondrial beta-oxidation is increased to compensate for the large amounts of FFA. For export, TGs become incorporated into packages of lipids and proteins called very low-density lipoproteins (VLDL). These particles consist of a core of TG, a phospholipid layer, and a protein layer of lipoproteins. This outer layer of large proteins is made of Apolipoprotein B (ApoB). ApoB is covalently modified with lipids during translation by Microsomal Triglyceride Transfer Protein (MTP). Mutations in MTP occur in NAFLD, resulting in a decreased export of TG via VLDL and hepatic steatosis [32].

If insulin resistance and hepatic steatosis is the first hit in the pathogenesis of NASH, the second hit is proposed to be a multifactorial process involving oxidative stress and lipid peroxidation, proinflammatory cytokines, adipokines and mitochondrial dysfunction. Many studies have demonstrated that oxidative
stress is a pathological feature of NASH [33]. Oxidative stress is an imbalance between pro-oxidants and anti-oxidants that leads to oxidative damage of cellular components. Reactive oxygen species (ROS) are these molecules, which have been produced by oxidative stress. In NAFLD, the oxidation of fatty acids is an important source of ROS. The degradation of FFAs in mitochondrial beta-oxidation leads to the formation of ROS such as superoxide anions. During the pathogenesis of NAFLD/NASH, mitochondria are severely impaired, termed mitochondrial dysfunction [34,35]. The damaged respiratory chain in mitochondria is the place in which ROS is generated. This is the result of alteration of electron transfer, leading to the reduction of respiratory complexes I and III and the production of ROS. The increase in beta-oxidation during NASH enhances electron delivery and blocks electron flow in the respiratory chain, resulting in the production of ROS [36]. In addition, the oxidation of glucose as seen in states of hyperglycemia and obesity can increase mitochondrial ROS production [33]. Other sources of ROS in the liver are CYP2E1, the microsomal cytochrome P450 2E1 enzyme and NADPH oxidase within Kupffer cells [37]. Interestingly, bacterial endotoxin from the gut increases Kupffer cell ROS production. Table 1 lists the sources of ROS, along with species of ROS.

The consequences of ROS on cells are DNA damage, amino acid oxidation, lipid peroxidation, cell necrosis, cell apoptosis, proinflammatory cytokine expression, hepatic stellate cell (HSC) activation, and fibrogenesis [38]. Lipid peroxidation is a process by which ROS oxidizes the unsaturated lipids within fatty hepatocytes. Lipid peroxidation products like aldehydes damage
hepatocytes to perpetuate the cycle of mitochondrial ROS production, resulting in hepatocellular apoptosis or necrosis [39]. These aldehydes, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA) damage the mitochondrial genome and impair the respiratory chain. Lipid peroxidation products can also impair respiratory chain component cytochrome c oxidase to affect electron flow in the respiratory chain, resulting in more ROS to perpetuate the cycle. Moreover, lipid peroxidation products can cause mitochondrial permeability and apoptosis [35]. ROS causes proinflammatory cytokines IL-6, IL-8, TNFα, and Fas ligand through the activation of NF-κB. TNFα can then damage mitochondrial DNA, exacerbating ROS generation [40]. Finally, ROS may deplete antioxidants in the liver. Low levels of glutathione (GSH) and decreased expression or activity of glutathione S-transferase in NASH would aggravate ROS-induced liver damage. In addition to ROS damage, ROS can induce hepatic stellate cell (HSC) activation, the myofibroblast-like cell of the liver, which causes fibrosis [31].
Table 1. Sources of Reactive Oxygen Species.

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>Source</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoplasmic Reticulum</td>
<td>CYP2E1, CYP4A10, CYP4A14</td>
<td>Species</td>
<td>Lipid Peroxidation</td>
</tr>
<tr>
<td>Plasma membrane enzyme complex</td>
<td>NADPH Oxidase</td>
<td>O(^{-})</td>
<td>4-HNE</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Mitochondrial Respiratory Chain</td>
<td>O(_2^{-})</td>
<td>MDA</td>
</tr>
<tr>
<td>Peroxisome</td>
<td>Acyl CoA Oxidase</td>
<td>OH(^{-})</td>
<td>H(_2)O(_2)</td>
</tr>
</tbody>
</table>
Contributions of Non-parenchymal Cells to NASH

In addition to oxidative stress and ROS-induced damage to hepatocytes, there are significant contributions to NASH pathogenesis from non-parenchymal cell populations. These include: liver-specific macrophages, the Kupffer cells (KC); sinusoidal endothelial cells and HSCs. Each of these cell types has a role in liver injury.

Kupffer Cells

KCs make up 80-90% of all tissue macrophages, while only 10% of total liver cell population [41]. They reside in the sinusoids, but can migrate to the space of Disse in order to mediate crosstalk of all liver cells. They are an important part of the innate immune system and are responsible for clearance of foreign particulate. They recognize exogenous material through pathogen-associated molecular patterns (PAMPs). KCs also recognize endogenous signals from damaged or dying cells through danger-associated molecular patterns (DAMPs). DAMPs include proteins such as heat shock proteins, high mobility group box 1 protein (HMGB1), extracellular matrix fragments, and non-protein molecules.

DAMPs signal through pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are part of a highly conserved family of PRRs, which play an essential role in the activation of KCs. The process of TLR-mediated activation of KCs in NAFLD/NASH includes TLR2, 4, and 9 signaling. TLR9 deficient mice have less steatohepatitis injury and fibrosis from the choline-deficient l-amino acid-defined (CDAA) diet-induced NASH. Interestingly, the production of IL-1β
was suppressed in TLR9 deficient mice [42]. Methionine-choline deficient diet (MCD) exacerbated steatohepatitis in TLR2 deficient mice [43], whereas TLR4 deficient mice are protected from steatohepatitis injury [44]. The PAMP lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria signals through TLR4, has been implicated in the pathogenesis of NAFLD [45]. Excess dietary intake of fructose and FFA contributes to altered gut flora motility, growth, and increased intestinal permeability, resulting in portal circulation of LPS [46]. Moreover, increased hepatocellular lipid accumulation contributes to altered KC function. The space occupied from the enlarged fatty hepatocytes may reduce sinudoidal perfusion, resulting in a number of pathologies. First, KCs will see more leukocytes and FFAs to initiate KC activation and inflammation. KCs in NALFD develop impaired mitochondrial function and increased apoptosis.

TLR ligand binding causes the assembly of the TLR signaling complex, including myeloid differentiation factor 88 (MyD88) and Toll-IL-1 receptor (TIR) domains of TIR domain-containing adaptor protein (TIRAP) or MyD88 adaptor-like (Mal). This leads to the activation of NF-κB and AP-1 transcription factors to produce cytokines such as IL-1 and IL-6. In addition, TLR may signal through a different complex called TIR domain-containing adaptor inducing interferon-β (TRIF) and its related adaptor molecule (TRAM), resulting in the production of interferon-β. Through the KC activation process, KCs produce and release cytokines, chemokines, enzymes, ROS, and nitric oxide. They recruit other immune cell types to the liver such as neutrophils, natural killer (NK) T cells, NK
cells, and other monocytes. Activated KCs phagocytose microorganisms, apoptotic cells, and cell debris from necroptotic cells and produce pro-fibrogenic factors and pro-inflammatory cytokines that activate hepatic fibrogenesis [41].

KCs also contribute to NAFLD/NASH through shifts in their polarization state, from alternatively activated, M2 polarized macrophages, and classically activated macrophages, M1 polarized macrophages [47]. The process of M2 polarization is dictated by cytokines from Th2 type T cells, IL-4 and IL-13, resulting in the expression of cell surface mannose receptor and arginase-1 [47]. The process of M1 polarization is caused by Th1 type T cell cytokines, interferon-gamma and TNFα [48]. The M2 macrophage is known as anti-inflammatory, pro-fibrogenic and immunosuppressive, while M1 is pro-inflammatory and associated with insulin resistance and obesity [49]. These activated macrophages can be shifted to and from the opposite polarization, depending on the state of the liver. Maina et al. demonstrated a bias towards M1 macrophages in mice on the MCD model of murine NASH [50]. In addition, polarization state may sensitize macrophages to second hits of NASH, such as ROS, resulting in increased production of cytokines and chemokines. Therefore, the polarization state is a very important contributor to the second hits that progress NASH.

*Liver Sinusoidal Endothelial Cells*

Liver sinusoidal endothelial cells (LSECs) make up the endothelial layer of the sinusoids. They are the first cells to come in contact with blood coming into the liver. Therefore, LSECs have specializations to help adapt to changes in their environment. One of these specializations is a structural characterization of
LSECs called fenestrae, which are open pores in the cells. These fenestrations filter fluids, solutes and particles from the blood, Space of Disse and the sinusoidal lumen [51]. Another function of LSEC fenestrae is endocytosis of particulates from the blood and sinusoidal lumen [52]. This contrasts with passive transport (transcytosis) of substances across the endothelium [53]. Endocytosis and transcytosis is bidirectional, from blood to lumen, and from lumen to blood. This feature is important to lipoprotein metabolism. Dietary fats are converted to chylomicrons in the blood and need to pass into the Space of Disse through LSECs before hepatocyte uptake [54,55]. In disease states, such as alcoholism, fenestration of LSECs is decreased, which is a mechanism of promoting hepatic steatosis in alcoholic liver disease [56]. Other roles of LSECs can be seen in hypoxia and endocytic shock [57], virus infection [58], cirrhosis [59] and liver cancer [60,61].

*Hepatic Stellate Cells and Fibrogenesis*

Hepatic fibrogenesis is a consequence of the activation of hepatic stellate cells [62] (Fig. 1.4). HSCs are quiescent retinoid–containing cells that reside in the space of Disse. They have many roles, contributing to hepatocellular function, hepatic regeneration, and response to injury. In response to specific signals, HSC can transform to a myofibroblast-like phenotype. This transformation is called HSC activation, and is a two-phase process: initiation and perpetuation. Initiation of HSC activation starts with cytokines and stimuli from the damaged liver, such as lipid peroxidation products and damaged hepatocytes. Perpetuation is the maintenance of the activated HSC phenotype, resulting in fibrosis. This is
balanced by resolution of fibrosis by reversion of activated HSCs to quiescent HSCs or by apoptosis.

Initiation begins with stimulation from neighboring cells such as endothelial cells, Kupffer cells, hepatocytes and platelets. Damaged sinusoidal endothelial cells secrete fibronectin and transforming growth factor-beta (TGF-β), both of which activate HSCs. Platelets stimulate HSC activation by platelet-derived growth factor (PDGF), TGF-β, and endothelial growth factor (EGF) [63]. Kupffer cells are a major source of HSC activation stimuli. Through the action of active TGF-β, ROS and lipid peroxides, HSCs are stimulated to proliferate, secrete matrix and lose their retinoids [1]. Hepatocyte sources of HSC activation include: lipid peroxides and hepatocyte apoptotic bodies [3,64].

Perpetuation is the process of prolonged HSC activation. Once activated, HSCs lose their retinoids and become myo-fibroblast-like cells. Activated HSCs proliferate, secrete extracellular matrix, are contractile, degrade matrix, undergo chemotaxis, lose their retinoid stores and cause inflammatory cell infiltration. These cell behaviors are caused by factors such as PDGF, Endothelin-1, TGF-β, matrix metalloproteinases and MCP-1 (Fig. 1.4).

Fibrogenesis is the process of HSC activation and generation of extracellular matrix. TGF-β released from damaged hepatocytes is a potent stimulus for HSC activation and matrix generation, primarily made up of collagen type I (COL1). COL1 is regulated tightly during transcription and post-transcriptional processing. TGF-β acts both extracellularly and intracellularly through signal molecules called Smads to initiate matrix production in HSCs.
TGF-β ligand binding to its receptor causes phosphorylation of Smads -2 and -3, resulting in Smad 2/3 translocation to the nucleus for transcription initiation.[65] TGF-β both suppresses hepatocyte proliferation, stimulates matrix production by HSCs and functions as an anti-inflammatory cytokine. The overexpression of TGF-β in mice results in fibrotic livers[65]. Fibrogenesis also includes matrix degradation. Early matrix degradation by activated HSCs is necessary for HSC chemotaxis and disrupts liver function when matrix replaces normal cells. Matrix metalloproteases (MMP) are the enzymes responsible for matrix degradation in the liver. However, during fibrogenesis, HSCs regulate their MMPs by tissue inhibitors of metalloproteinases (TIMPs). These enzymes inhibit the MMPs to reduce the matrix degradation during fibrosis, resulting in fibrosis [66].
Figure 1.3 HSC activation phenotypes. HSC activation is a response to tissue injury. This is a transition from quiescent retinoid-containing cells to proliferative, contractile and fibrogenic phenotypes of a myofibroblast. Phenotypic changes of an active HSC include, retinoid loss, proliferation, contractility, fibrogenesis, along with matrix degradation, chemotaxis, and chemoattraction. Specific signals that regulate HSC activation phenotypes include, TGFβ, PDGF, MMP and MCP-1. Activated HSCs may revert to a quiescent phenotype of undergo apoptosis.
IV. Interleukin-1, Caspase-1 and Inflammasome

Interleukin-1

The family of interleukin-1 includes IL-1α, IL-1β and IL-1 Receptor Antagonist. IL-1β plays a role in a wide variety of inflammatory diseases, such as cancer, infection, and autoimmune disorders. Increasing levels of IL-1β have been correlated with diseases like rheumatoid arthritis, gout, and trauma. Inflammatory diseases such as Muckle-Wells and Familial Mediterranean Fever are characterized by increased levels of IL-1β [67]. Some of these disorders can be treated with IL-1 Receptor Antagonist [68]. Elevated levels of IL-1β for even a short duration cause many symptoms of toxicity, including fever, hypotension and production of other cytokines like IL-6 [69,70]. Therefore, it is important to tightly regulate the production and release of IL-1β.

There are many means to regulate IL-1β, including its interaction with its receptor. These strategies employ the use of decoy receptors and receptor antagonist [71]. The IL-1β receptor acts as a dual receptor for IL-1α, but signals in the same manner. Once either IL-1β or IL-1α binds the receptor, an intracellular Toll-like and IL-1R (TIR) domain recruits scaffolding molecules. Next more IL-1β, IL-6 and other proteins like collagenases are produced [72]. Another way of regulating IL-1β is through transcription via AP-1 and NF-κB. IL-1β is transcribed after stimulation of the IL-1R by TNF, complement factor C5a, hypoxia or LPS. Translational control is simple because IL-1β is produced
on ribosomes in the cytosol. IL-1β is synthesized as a zymogen of 31 kDa, and is called pro-IL-1β. This form is inactive until it is cleaved into the mature 17kDa IL-1β form. Pro-IL-1β is cleaved by the interleukin-1 converting enzyme (ICE), also referred to as caspase-1 [73].

*Interleukin-18*

Another family member of IL-1 is interleukin-18 (IL-18). IL-18 was first described as an interferon-gamma (IFNγ) –producing cytokine [74]. IL-18 is produced by macrophages and hepatocytes. Its binds to the IL-18 receptor (IL-18R) on T cells, natural killer cells and neutrophils [75]. IL-18 is implicated in inflammatory diseases, such as adenomyosis [76], Hashimoto’s thyroiditis [77] and Alzheimer’s disease [78].

*Caspase-1*

*Caspase-1, the protein*

ICE, or caspase-1 is a cysteine aspartic protease that is responsible for the cleavage of IL-1β and IL-18. It is a pro-inflammatory caspase, along with human caspases 4, 5, 12 and mouse caspase-11 [79]. Caspase-1 is highly expressed in spleen, lung, monocytes and macrophages. It is synthesized as an inactive zymogen of 45 kDa [80]. The active form of caspase-1 is a heterotetramer consisting of two 20 kDa and two 10 kDa subunits. The 20 kDa subunit, termed p20 is the catalytic domain, although both the p20 and p10 subunits are needed for IL-1β cleavage [79]. Once activated, the p20 and p10 subunits are used as a
measure of caspase-1 activity, but are difficult to detect intracellularly because they are often secreted out of the cell [81]. Caspase-1 also has a role in non-traditional protein secretion, such as that of IL-1α and fibroblast growth factor-2, even though they are not direct cleavage targets of caspase-1 [82].

Regulation

Caspase-1 is regulated by a variety of activators and inhibitors. Activators include substances like crystals, toxins and pathogenic antigens. These are the same activators that cause IL-1β activation, such as the influx of intracellular K⁺ and ATP-gated P2X7R [80]. LPS is a potent stimulator of caspase-1 in monocytes, but differs in macrophages [83]. Proteins of the CATERPILLAR family of scaffolding proteins, which denotes CARD, transcription enhancer, R-binding, pyrin, lots of leucine repeats can also activate caspase-1 [84]. These family members include Ipaf-1, which contains a Caspase Recruitment Domain (CARD) to interact with the CARD on caspase-1 for its activation; PYPAF5 has a pyrin domain capable of activating caspase-1; and Nod1, which contains a CARD to interact and activate caspase-1. Another protein activator of caspase-1 with a CARD domain is Rip2/CARDIAK. Rip2 [85]. Rip2 is a receptor interacting protein that plays a role in NF-κB and caspase-1 activation [85]. Invasive bacteria and pathogenic materials play a role in the typical innate immune response of activating capsase-1. Muramyl dipeptide, MDP, a component of bacterial peptidoglycan activates caspase-1 in human monocytes [86]. Salmonella typhimurium targets caspase-1 –mediated cell death through Ipaf [87]. Shigella infected macrophages, and the cause of bacillary desentary activates
caspase-1 [88,89]. Toxins, such as anthrax lethal toxin induces caspase-1 – mediated necrosis of macrophages [90]. Crystals, such as silica, and those in joint inflammation, like monosodium urate acid crystals and calcium pyrophosphate dihydrate activate caspase-1 [91].

Inhibitors of caspase-1 include viral proteins and chemical inhibitors. The first identified inhibitor of caspase-1 is cytokine response modifier A, or CMA, which is a product of cowpox virus [92]. There are several other viral inhibitors of caspase-1, which include p35, p49, OpIAP, DIAP1 and others that do not directly inhibit caspase-1 [92]. Proteins containing a CARD domain, such as Pseudo-ICE and ICEBERG share a large percentage of homology with caspase-1 and block the interferon-gamma and LPS –induced IL-1β release from monocytes [93]. Card only protein, or COP is a protein inhibitor of caspase-1 by binding to the pro-domain of caspase-1 and blocking the production of IL-1β [94]. Another family of proteins that act as caspase-1 inhibitors is that of Bcl, namely Bcl-2 and Bcl-XL, which suppress caspase-1 and IL-1β from macrophages treated with MDP [95]. Besides proteins, most of the well-known inhibitors of caspase-1 are those of peptides that correspond to the cleavage sites of caspase-1 target proteins. These chemical inhibitors act as pseudosubstrates for caspase-1, and thus confer competitive inhibition [96].

*The inflammasome complex*

The activation of caspase-1 requires the formation of a multimeric complex, termed the inflammasome. The inflammasome complex signals via
sensing PAMPs and DAMPs by intracellular nucleotide-binding domain leucine-rich repeat–containing receptors (NLR), which is similar to TLRs. The NLR family consists of NLRP-1 to NLRP-14, Ipaf, and Nod proteins [97]. These proteins form a common platform upon which the inflammasome assembles. In order to assemble with other proteins, the NLRs have domain structures upon which interact with adaptors and caspase-1. The NLRPs contain an N-terminal pyrin domain (PYD), and are NLR members. To interact directly with caspase-1, NLRs known as NLRCs have a CARD domain [98]. The most well studied inflammasomes are NLRP1, NLRC3, NLRP4 and AIM2. All inflammasomes act as pro-inflammatory caspase activating platforms, but are assembled by different stimuli. NLRP1, NLRC3 and NLRP4 assemble upon various stimulators, whereas AIM2 is a DNA sensing inflammasome [98]. Another component of the inflammasome is apoptosis-associated speck-like protein containing a CARD (ASC). ASC is an adaptor protein present in NLRP3 and AIM2 inflammasomes that helps recruit caspase-1 to the inflammasome for activation [99]. Inflammasome assembly upon stimulation is illustrated in Figure 1.5.
**Fig 1.4. Mechanisms of Inflammasome Assembly and Caspase-1 Activation.** IL-1β, LPS, and other PAMPs activate IL-1R and TLR4 receptors, resulting in NFκB translocation to the nucleus, which initiates pro-IL-1 and inflammasome component transcription. A second signal is required for the assembly of the inflammasome, including PAMPs such as bacterial products, ROS, lysosomal damage and cathepsin b release and potassium efflux. Once the NLRP3 inflammasome has formed, pro-caspase-1 is cleaved into the active caspase-1, which can cleave its target, pro-IL-1β into IL-1β and its subsequent release.
Hepatic Inflammasome

In the past few years, the inflammasome has been the topic of intense investigation in obesity and metabolic disturbances, such as insulin resistance and NASH. A majority of the investigations done on obesity and the inflammasome have explored adipose tissue and insulin resistance. Vandamagsar et al. demonstrate that Nlrp3 inflammasome plays a role in obesity-induced adipose tissue inflammation and insulin resistance [100]. Mice on a high fat diet have increased expression of adipose caspase-1 [100,101]. Caspase-1 is expressed in both adipose tissue and liver. Csak et al. demonstrate that caspase-1 in hepatocytes is activated upon MCD feeding [102]. Mice deficient in inflammasome components ASC and Nlrp3 are protected against high fat diet-induced steatosis [101]. Similarly, Casp1-/- mice on a high fat diet had a reduction in weight gain and improved insulin senstivity [103]. These studies demonstrate that the inflammasome has powerful effects on adipose and liver, especially on glucose homeostasis. However, there still remains a lack of evidence for the role of caspase-1 in diet-induced steatohepatitis. These models have only investigated obesity and insulin resistance, while leaving mechanisms of caspase-1 in inflammation, HSC activation and fibrogenesis unexplored.

V. Mouse Models of NASH

There are many different ways of modeling NASH in mice. The problem is that not one of them is the right mix of both pathology and metabolism that recapitulates human NASH encompassing insulin resistance and fibrosis.
Commonly used diet models resemble either NASH histopathology, pathology, but not both. A combination of genetics and diets are often utilized as well. The model of histopathology is the MCD diet, which results in inflammation and fibrosis similar to severe human NASH. The mechanism of action is limiting methionine and choline needed in mitochondrial fatty acid oxidation and the secretion of FA out of the liver in the form of very low-density lipoprotein. It also removes the protection of glutathione to second hits like oxidative stress [104]. This model lacks the etiology of NASH, insulin resistance and obesity. Mice on the MCD diet can lose up to 40% of their body weight, making this model fairly questionable for replicating the pathophysiology of NASH. A model that does result from the pathology of insulin resistance and obesity is the high fat diet, otherwise called the western diet model. This diet consists of a mixture of saturated and unsaturated fat (10-40%), cholesterol, sucrose and carbohydrate. The high fat diet results in increased inflammation, oxidative stress, hepatic lipogenesis, and leptin and decreased adiponectin [105]. The high fat diet can be a variable model of NASH, based on length of feeding protocol, species of animal on the diet, fat or carbohydrate content of the diet as well as the type, or “quality” of fat. The intragastric high fat overfeeding paradigm closely resembles human NASH, but is a difficult technique to use long-term. Long-term studies of the high fat diet are more widely accepted [106]. Diets can exacerbate an existing phenotype of genetically altered mouse NASH models, so they are often combined into one protocol. There are many naturally occurring genetic mutations, that when paired with a diet, lead to NASH. These include ob/ob, leptin deficient
mice or db/db, leptin receptor deficient mice combined with the MCD diet. The db/db mice on the MCD diet had increased ALT levels and inflammatory cytokine expression than ob/ob mice, making db/db a more attractive model for NASH than ob/ob. Combinations of genetic mutations and the high fat diet include: fa/fa, leptin receptor deficient rats and foz/foz, mice with a dysfunctional Alms1 gene. Targeted gene mutations also make animal models of diet-induced NASH feasible. These include mice with the following genes knocked out: PPARα, Cyp2e1, TNFα, iNOS, TNFR1, JNK1 or JNK2, Osteopontin and apoprotein E [104]. While these models are useful for answering specific questions about NASH pathogenesis, not one of them encompasses all of the characteristics of human NASH. Therefore, there is a great need for the development of such a model.

The following chapters will explore the activation of caspase-1 in diet-induced murine NASH. Chapter 2 discusses methods of caspase-1 expression, activation and the evaluation of mice phenotypes on the diets. Chapter 3 investigates the role of caspase-1 in the MCD diet model of NASH. Additionally, Chapter 4 explores the role of caspase-1 in early high-fat diet-induced NASH. In Chapter 5, preliminary data regarding the induction of caspase-1 in hepatic stellate cells is discussed as well as questions further exploration that would result in an understanding of molecular mechanisms of caspase-1 activation in NASH pathogenesis.
Animal Studies. These experimental protocols were approved by the Institutional Animal Care and Use Committee at the Cleveland Clinic. Male C57BL/6 mice, 20 to 25 g of body weight, were purchased from Jackson Laboratory, Bar Harbor, ME. Caspase-1 knockout mice (Casp1<sup>-/-</sup>) (provided by Dr. Richard Flavell from Yale University, New Haven, CT) were described previously and backcrossed at least 10 generations to a C57BL/6 background [107,108]. Mice were housed in microisolator cages in 10 hours light and 14 hours dark. Mice were placed on a methionine and choline deficient (MCD) diet (TD 90262, Teklad Mills), which has been extensively shown to result in steatosis associated with significant inflammation and progressive fibrosis pathologically similar to human severe steatohepatitis [109,110]. In this model steatosis results from both decreased mitochondrial oxidation of fatty acids and decreased export of fatty acids in the form of very low density lipoprotein [104]. Identical groups of animals (n = 5 - 7 in each group) received a standard diet consisting of 5% fat (TD 2918, Teklad Mills, Madison, WI) to act as controls (CTL). Total body weight was measured at 0, 1, 3 and 6 weeks. Food was removed 5 hours prior to kill for fasting. Animals in each group were sacrificed after 6 weeks on respective diets. In selective studies, C57BL/6 male mice of 20-25 g in weight were placed on the MCD diet for the intravenous injection of liposomes encapsulating phosphate (PBS) or clodronate (CLOD) (n=3-7 in each group). After 5 weeks of MCD diet, animals
were injected twice 5 days apart with 0.1 ml per 10 g of body weight of a 1mg/ml suspension of liposomes as previously described[111]. Animals in each group were sacrificed after 6 weeks on respective treatments.

In two separate feeding trials, mice were placed on a high fat diet (HFAT) containing 42% kcal from fat (TD 88137 Teklad Mills, Madison, WI) or a standard diet (CTL) containing 5% fat (TD 2918, Teklad Mills, Madison, WI). Body weight was measured at 0, 1, 3, 6, 9, and 12 weeks. One group of animals were sent to Case Western Reserve University for body fat imaging at week 10, housed a week in their facility, and transported back on week 12, 3 days before euthanasia. Animals in each group were euthanized after 12 weeks on respective diets.

**Imaging of Animals for Body Fat Analysis.** Each animal was initially anesthetized with 2-3% isoflurane in oxygen. The animals were then placed in a prone position within a Bruker Biospec 7T MRI scanner (Bruker Biospin, Billerica, MA). A 72-mm diameter volume coil was used for excitation and signal detection to maximize the uniformity of the images. After localizer scans, a Relaxation Compensated Fat Fraction (RCFF) MRI acquisition and reconstruction process was used to generate quantitative fat fraction maps for each imaging slice [112]. Briefly, three asymmetric echo spin echo MRI acquisitions were acquired (TR/TE=1500ms/20ms, 17-25 coronal slices, resolution = 200um x 200um x 1000 um, 2 averages). The three acquisitions were acquired with different echo shifts to allow separate fat and water images to
be generated. Finally, a semiautomatic image analysis was performed to segment and calculate the volumes of peritoneal and subcutaneous adipose tissues, respectively.

**Histopathology, Immunostaining and Serum Assays.** Blood and liver tissue were collected after a 5-h fast. Plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity was measured using a commercial kit (Diagnostic Chemicals). Liver tissue was fixed in 10% Formalin and embedded in Tissue Path (Fisher Scientific, Pittsburgh, PA). Fresh liver tissue was frozen in OCT. Hematoxylin and eosin liver specimens were evaluated by light microscopy. Liver triglyceride determinations were measured biochemically using the Triglyceride Reagent Kit from Pointe Scientific Inc (Lincoln Park, MI). Steatosis, inflammation, and ballooning were assessed in the livers of mice on the HFAT and CTL diets by an experienced pathologist, Bettina G. Papouchado (BGP) in a blinded fashion. Steatosis, inflammation, and ballooning were scored based on NAFLD activity score (NAS) [113].

**Cell Lines and Culture.**

Primary mouse hepatocytes and total non-parenchymal cells were isolated from C57BL/6 mice on a 6-week MCD diet using the method of Seglen [114]. The mice were anesthetized and the livers were perfused through the portal vein with warm, oxygenated Hanks (-) with 1mM EGTA and 10mM HEPES followed by Williams E media containing 10mg collagenase per mouse. Livers were minced
and hepatocytes were collected after centrifugation. Resulting cell suspensions were used to collect total non-parenchymal cells. Total non-parenchymal cells were isolated by a 40% to 70% Percoll gradient.

**Immunoblot, Immunoprecipitation Analysis, and ELISA.** 500 µg of total liver protein was immunoprecipitated with 5 µg rabbit anti-caspase-1 antibody. Immunoblot analysis was performed using 30-40 µg whole liver lysate, 20 µg hepatocyte or non-parenchymal fraction, or immunoprecipitated lysate. Whole liver lysate and hepatocyte/non-parenchymal samples were resolved by 12-15% SDS-PAGE and immunoprecipitated liver lysate samples were resolved by a 4-20% gradient gel (Invitrogen), transferred to nitrocellulose membrane, and blotted with appropriate primary antibodies. The membrane was incubated with peroxidase-conjugated secondary, and the bound antibody was visualized using a chemiluminescent substrate (Amersham Biosciences or GE Healthcare) and Kodak X-OMAT film (Eastman Kodak, Rochester, NY) or in high fat studies with super ECL (GE Healthcare) and Eastman Kodak Co. Image Station 4000R. The following primary antibodies were used: rabbit anti-Caspase-1 (Millipore, Billerica, MA), rabbit anti-IL1β (Abcam, Cambridge, MA); rabbit anti-apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (Abcam, Cambridge, MA); rabbit anti-alpha smooth muscle actin (Abcam, Cambridge, MA). Lysates were probed for IL-1β with IL-1β ELISA Ready-SET-Go (eBioscience, San Diego, CA). IL-1β was normalized to lysate protein concentration.
**Assessment of hepatic caspase-1 activation and cellular localization.** Caspase-1 activity was determined using 200 µg whole liver protein with Caspase-1 Fluorometric Assay Kit (Abcam, Cambridge, MA). Immunohistochemistry for caspase-1 was performed using paraffin embedded liver tissue using standard DAB technique. The following primary antibodies were used: rabbit anti-Caspase1 (Millipore, Billerica, MA).

**Apoptosis Assessment.** Frozen tissue sections (4 µm) were prepared, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed following manufacturer’s instructions (in situ cell death detection kit; Roche Molecular Biochemicals, Mannheim, Germany). Caspase activation was quantified by immunostaining for active caspase 3 using a cleaved caspase-3 antibody (Cell Signaling). Hepatocyte apoptosis in liver sections was quantitated by counting the number of TUNEL-positive or active caspase 3 –positive cells in 5 random microscopic fields (40x), as previously described [115].

**Real-time PCR.** Total RNA was isolated from liver tissue or HSC using RNeasy Tissue Mini kit (Qiagen, Valencia, CA). The reverse transcript was synthesized from 1 µg of total RNA using SuperScript cDNA synthesis kit (Ambion, Grand Island, NY). Real-time PCR quantification was performed. 25 µl of reaction mix contained: cDNA, Syber Green buffer, Gold Taq polymerase, dNTPs, and primers at final concentrations of 200 µm. RT-PCR was performed in the Mx3000P cycler.
(Stratagene): 95 °C for 10 min, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C followed by 1 min at 95 °C, 30 s at 55 °C and 30 s at 95 °C. The relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those of 18s, using MxPro 4.10 software (Stratagene, Santa Clara, CA). Primers are listed below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5'</th>
<th>Reverse 5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>ACGGAAGGGCACCACCAGGA</td>
<td>CACCACCACCACCGGAATCG</td>
</tr>
<tr>
<td>ASC</td>
<td>CTGTGTCAGGGGATGAACTCAA</td>
<td>GCCATACGACTCCAGATAGTC</td>
</tr>
<tr>
<td>Casp1</td>
<td>ACAAGGCACCGGGACCTATG</td>
<td>TCCCAGTCAGTCTCGGAATG</td>
</tr>
<tr>
<td>CRP2</td>
<td>GCTACGGGAAAGATGATTGACC</td>
<td>CTCAGTCAGTTTATGACTCC</td>
</tr>
<tr>
<td>Col11α1</td>
<td>ATGTTCAGCTTTGTGGACCTC</td>
<td>CAGAAACACAGACTG</td>
</tr>
<tr>
<td>F4/80</td>
<td>CCCCAGTGCTCCTTACAGAGTG</td>
<td>CTGGATAGCTTCTTCTGCTG</td>
</tr>
<tr>
<td>IL-1</td>
<td>CCAGGATAGGACCCCAA</td>
<td>TCCCGACCATTGCTGTT</td>
</tr>
<tr>
<td>IL-6</td>
<td>TAGTTCCCTCCTACCCCAATTTCC</td>
<td>TTGGTGCTCTAGCCACTCCTC</td>
</tr>
<tr>
<td>IL-8</td>
<td>GCCCCCAGACAGAACTCAGTAG</td>
<td>AGCCTTGCTTTTATGACTAC</td>
</tr>
<tr>
<td>IL-18</td>
<td>GACTCTTGGCTCAGCTTCAAGG</td>
<td>CAGGCTGTCTTTGTGCAACGA</td>
</tr>
<tr>
<td>Mcp1</td>
<td>AGGTCTCCTGATCTGGTCTC</td>
<td>TCTGGACCCATTGCTC</td>
</tr>
<tr>
<td>Mmp13</td>
<td>CTCCCTTTCCTGACGCTCGACTC</td>
<td>CTGGGAGTTGACTGACTG</td>
</tr>
<tr>
<td>Nlph3</td>
<td>ATGTTGCAGCCAGAAAGGGG</td>
<td>CATGAGTGTGGCTAGATCA</td>
</tr>
<tr>
<td>Ppara</td>
<td>AGAGGCCATCTGCTCTTCTC</td>
<td>ACTGGTAGCTGCAAACAA</td>
</tr>
<tr>
<td>Scl1</td>
<td>CCCGAGACCCCTAGATCGTCA</td>
<td>TAGCCTGTGAAAAAGTTTCTCGGAAACC</td>
</tr>
<tr>
<td>αSma</td>
<td>GTCCACAGACATCAGGAGTAAA</td>
<td>TCGGATACTCCACGGCTCAGGA</td>
</tr>
<tr>
<td>Srebpl</td>
<td>AACGTCACTTCCAGCTACGAC</td>
<td>CCAGTGAGTGGCTACAGAGC</td>
</tr>
<tr>
<td>Temp1</td>
<td>GCAACTCGGACCTGGTCATAA</td>
<td>CGGCCGGTGATGAAACT</td>
</tr>
<tr>
<td>TGF-β</td>
<td>CTCGCGTGGTCTCAGTG</td>
<td>GCCTTAGGTTGACAGGATCTG</td>
</tr>
<tr>
<td>Tnfa</td>
<td>ATGAGCACAGAAAGCATGATC</td>
<td>TACAGGCTTGTGACTGAAAT</td>
</tr>
</tbody>
</table>

**Statistical Analysis.** Values reported are means ± SEM. The data for the MCD study were analyzed for normality using the D’Agnostino & Pearson omnibus normality test. If the data were normally distributed, One way ANOVA was done for the analysis of three or more groups and the student’s t-test was used for two
groups. If the data was not normally distributed we used the Kruskal-Wallis nonparametric test. Data for the high fat study were analyzed by ANOVA, using the general linear models procedure (SAS, Carey, IN). The Shapiro-Wilk test was done for normality. Data were log transformed if needed to obtain a normal distribution. Least square means testing was performed for follow-up multiple comparisons. Non-parametric Mann-Whitney-U test was performed for analysis of histopathological NAFLD activity score between wildtype mice on the high fat and Casp1−/− on the high fat diet groups. Differences were considered to be statistically significant at p<0.05.
CHAPTER 3
Caspase-1–mediated regulation of fibrogenesis in diet-induced steatohepatitis

Portions of this chapter are published in the Journal of Laboratory Investigation, 92(5).

Summary

Nonalcoholic steatohepatitis (NASH) is typically associated with pro-apoptotic caspase activation. A potential role for pro-inflammatory caspases remains incompletely understood. Our aims were to examine a potential role of caspase-1 in the development of liver damage and fibrosis in NASH. C57BL/6 wild type (WT), developed marked steatohepatitis, HSC activation, fibrosis, and increased hepatic caspase-1 and IL-1β expression when placed on the methionine-choline deficient (MCD) diet. Marked caspase-1 activation was detected in the liver of MCD-fed mice. Hepatocyte and non-parenchymal fractionation of the livers further demonstrated that caspase-1 activation after MCD feeding was mainly localized to non-parenchymal cells. Caspase-1-knockout (Casp1−/−) mice on the MCD diet showed marked reduction in mRNA expression of genes involved in inflammation and fibrogenesis (TNFα was 7.6–fold greater in WT vs. Casp1−/− MCD-fed mice; F4/80 was 1.5-fold greater in WT vs. Casp1−/− MCD-fed mice; α-SMA was 3.2-fold greater in WT vs. Casp1−/− MCD-fed mice; Collagen 1-alpha was 7.6 -fold greater in WT vs. Casp1−/− MCD-fed mice; TGFβ was 2.4–fold greater in WT vs. Casp1−/− MCD-fed mice; CRP2 was 3.2-fold greater in WT vs. Casp1−/− MCD-fed mice). Furthermore, Sirius red staining for hepatic collagen deposition was significantly reduced in Casp1−/− mice MCD-fed mice compared to
WT MCD-fed animals. However, serum aminotransferase (ALT) levels, caspase 3 activity and TUNEL positive cells were similar in Casp1−/− and WT mice on the MCD diet. Selective Kupffer cell depletion by clodronate injection markedly suppressed MCD-induced caspase-1 activation and protected mice from fibrogenesis and fibrosis associated with this diet. Conclusion: this study uncovers a novel role for caspase-1 in inflammation and fibrosis during NASH development.

**Introduction**

Nonalcoholic Fatty Liver Disease (NAFLD) is currently the most common form of chronic liver disease affecting both adults and children, and is strongly associated with obesity and insulin resistance [14,15]. One in three adults and one in ten children or adolescents in the United States have hepatic steatosis, a stage within the spectrum of NAFLD, that is characterized by triglyceride accumulation in liver cells and follows a benign non-progressive clinical course [116,117]. Nonalcoholic steatohepatitis (NASH) is defined as lipid accumulation with evidence of cellular damage, inflammation and different degrees of scarring or fibrosis [118]. NASH is a serious condition as approximately 25% of these patients progress to cirrhosis and its feared complications of portal hypertension, liver failure and hepatocellular carcinoma [119,120,121]. The pathogenesis of NAFLD/NASH in particular the mechanisms responsible for liver injury and disease progression remains incompletely understood but are of significant biomedical importance as identification of these processes may help to identify
novel diagnostic and therapeutic targets for this highly prevalent and potentially serious disease.

Since the original description that caspase activation and hepatocyte apoptosis are characteristic pathologic features in the liver of NASH patients [122], a growth of data have demonstrated a key role for caspase-dependent cell death in NASH pathogenesis [115,123,124,125]. Caspases are a family of cysteine proteases with unique substrate specificities that play a central role in the apoptotic machinery [126,127]. They are synthesized as inert zymogens and upon receipt of apoptotic stimuli, cells activate initiator caspases such as caspase-1, 2, 8, 9, and 10 that, in turn, proteolytically cleave and activate effector caspases including caspase 3, 6, and 7. Caspases have been further categorized as either proinflammatory or proapoptotic, depending upon their participation in these cellular programs. The proinflammatory caspases include caspase-1, 11 and 12 in mouse and caspase-1, 4, and 5 in human [128]. Targeting caspase activity and apoptosis has gained significant attention for developing of novel therapeutic diagnostic strategies for NASH patients. Recent data suggest that pan-caspase inhibition protects again diet-induced steatohepatitis in different dietary murine models [129,130,131]. These pan-caspase inhibitors, not only inhibit caspase-mediated cellular apoptosis, but also block the caspase-1-dependent processing and activation of various proteins with functions in inflammation and tissue repair during tissue damage. However, the contribution of caspase-1-dependent processes to liver injury and fibrosis remains unclear. In the present study we examined the occurrence and significance of caspase-1 activation in NASH.
Results

Hepatic Caspase-1 activation is a prominent pathological feature in experimental NASH. To investigate the role of caspase-1 activation in the pathogenesis of NASH, we initially placed C57BL/6 mice on the methionine and choline deficient (MCD) diet, which has been extensively shown to be associated with progressive fibrosing steatohepatitis pathologically similar to human severe steatohepatitis (22, 23). After 6 weeks on the respective diets we observed significant hepatic fat accumulation induced by MCD feeding (Fig. 2A), in conjunction with an increased in histological parameters of liver injury including hepatic inflammation, and hepatocyte ballooning (Fig. 2A). Expression of caspase-1 was significantly increased in mice on the MCD feeding compared to controls (Fig. 2B), This was accompanied by increased mRNA levels of ASC, a key component of the inflammasome, the protein platform that activates caspase-1, as well as IL-1 but not of IL-18 (Fig. 2B). These changes in the livers of mice fed the MCD diet were associated with a marked increased in inflammasome activation (Fig. 2C and D), as well as caspase-1 protein expression and activity levels (Fig. 2E - G). Consistent with these data, liver active mature IL-1β protein expression was also significantly increased in MCD-fed animals compared to CTL mice (Fig. 2H and I). Immunohistochemical analysis of liver sections from the two groups of mice showed that caspase-1 was primarily localized to non-parenchymal sinusoidal cells, and to a lesser extent to hepatocytes (Fig. 2J). This was further confirmed by fractionation of the liver tissue from MCD fed animals
into hepatocytes and total non-parenchymal cells further demonstrating that caspase-1 and IL1β protein expression was markedly enhanced in the non-parenchymal fraction compared to hepatocytes (Fig. 2K).
Fig. 2. Hepatic caspase-1 activation during diet-induced steatohepatitis. (A) C57BL/6 mice were placed on a methionine and choline deficient (MCD) diet or a control (CTL) diet for 6 weeks (n = 5-7 in each group). Representative microphotographs of Hematoxylin & Eosin (H&E) staining from mice on the two groups (40x). (B) RT PCR analysis of inflammasome components apoptosis speck-like protein containing a CARD (ASC), NACHT, LRR and PYD domains-containing protein 3 (NALP3), caspase-1 (CASP1), interleukin-1 (IL-1) and interleukin-18 (IL-18) in liver WT mice (n=4 in each group). (C) Western blot of ASC on caspase-1 immunoprecipitated whole liver lysates in WT MCD-fed mice compared to WT CTL-fed mice and (D) densitometric analysis of ASC (n=4). (E) Western blot of caspase-1 on whole liver lysates in WT MCD-fed mice compared to WT CTL-fed mice and (F) corresponding densitometric analysis to GAPDH (n=4). (G) Caspase-1 activity assay was performed on whole liver lysates of WT MCD-fed mice and compared to CTL-fed mice (n=9 in each group). (H) Western blots of IL-1 displays pro IL-1 processing into active IL-1β in whole liver lysates of MCD-fed mice compared to CTL-fed mice and corresponding (I) densitometric analysis to GAPDH. (J) Representative microphotograph of caspase-1 immunohistochemistry in paraffin-embedded liver sections of MCD-fed mice compared to CTL-fed animals (Magnification 40x). (K) Western blot of caspase-1 and IL-1 was performed on cell lysates from isolated primary hepatocytes and total non-parenchymal cells from MCD-fed animals. Results are expressed as mean ± S.E.M. * P < 0.05; ** P < 0.01; compared to controls.
**Caspase-1 suppression is associated with dissociation between hepatic triglyceride accumulation and inflammatory activity.** Having established the presence of inflammasome activation and increased caspase-1 activity in the liver of MCD fed mice, we next sought to investigate whether caspase-1 participates in NASH development using caspase-1 knock out mice (*Casp1*<sup>−/−</sup>). We first investigated whether *Casp1*<sup>−/−</sup> mice are resistant to hepatic steatosis and inflammation. C57BL/6 wild type, Casp1 knock out mice were placed on either a MCD diet, or control (CTL) diet (n = 5 - 7 in each group) for 6 wks. WT mice and *Casp1*<sup>−/−</sup> mice had similar weight changes on the MCD-diet (Fig. 3A). However, microscopic examination of H&E and oil red-O staining showed that *Casp1*<sup>−/−</sup> mice on the MCD-diet developed more significant macrovesicular hepatic steatosis compared to the WT mice on this diet (Fig. 3B, C). Consistently with these results, hepatic triglyceride levels were significantly more elevated in *Casp1*<sup>−/−</sup> mice compared to WT mice on the MCD diet (Fig. 3D). Histological examination of liver samples of other individual features associated with NASH demonstrated a decrease in inflammatory foci resulting in lower inflammatory activity scores in the *Casp1*<sup>−/−</sup> mice on the MCD diet compared to WT mice on this diet. However, the total NAFLD activity score (NAS) was similar in the two groups of mice as mainly as a result of higher degree of steatosis as well as higher levels of hepatocyte ballooning present in MCD-fed *Casp1*<sup>−/−</sup> mice. Serum ALT levels were similarly elevated in *Casp1*<sup>−/−</sup> mice and WT mice on the MCD diet compared to animals fed the control diet (Fig. 3E). We next examined the inflammatory state of the liver at the molecular and cellular level, and found a
marked reduction in mRNA levels of TNF-α, F4/80 and CD11c in the MCD-fed

Casp1−/− mice compared to the WT animals on the MCD diet (Fig. 3F, G, H).
Fig. 3. **Caspase-1 suppression is associated with dissociation between hepatic triglyceride accumulation and inflammatory activity in diet-induced steatohepatitis.** (A) Body weight was measured at weeks 0, 1, 3, and 6 of WT and Casp1−/− mice on a 6-week CTL or MCD diet (n = 5-7 in each group). (B, C) Representative images of H&E and Oil red O staining (ORO) staining of liver tissue displays the severity of cellular injury, further investigated by: (D) hepatic triglyceride (TG) (n=4-5) and (E) serum alanine aminotransferase (ALT) levels (n=5-6). (F) RT PCR analysis of pro-inflammatory cytokine tumor necrosis factor (TNFα) (n = 3-4), (G) macrophage marker F4/80 (n=6-8) and CD11c (n=4-5) in liver of Casp1−/− mice compared to WT mice. Results are expressed as mean ± S.E.M. * P < 0.05; ** P < 0.01; ***P < 0.001 compared to controls.
HSC activation and collagen deposition induced by the MCD diet are decreased by caspase-1 suppression independent of caspase 3 activation and apoptosis. The findings of a key role of caspase-1 in hepatocyte injury and inflammatory signaling, two events that have been linked to HSC activation, led us to further examine the role of caspase-1 in fibrogenesis and fibrosis induced by the MCD diet. While, as expected after six weeks on the MCD diet wild type animals showed a marked increase in the mRNA expression of genes involved in HSC activation and fibrogenesis, such as TGFβ, αSMA, COL1A1, and CRP2 (Fig. 4A -D). These changes were significantly reduced in the Casp1−/− MCD-fed mice. In addition, αSMA protein expression was increased in WT MCD-fed mice, while it was significantly reduced in Casp1−/− MCD-fed mice (Fig. 4E, F). More importantly, an almost four-fold increase in collagen deposition as demonstrated by Sirius red staining of liver tissue coupled to quantitation by digitized image analysis was present in WT animals on the MCD diet compared to the wild type animals on the control diet (Fig. 4G, H), while these increases in collagen deposition were significantly less prominent in the Casp1−/− mice although still higher than in mice on the CTL diet (Fig. 4G, H). We next quantified the amount of hepatocellular cell death present in the various groups of mice. Caspase 3 activation and TUNEL-positive hepatocytes were increased to a similar extend in both WT animals and Casp1−/− mice on the MCD diet compared to animals on the CTL diet (Fig. 5A – D). Taken together, these observations suggest that during NASH development, caspase-1 activation in hepatocytes plays an important role
in hepatocellular injury, inflammatory signaling, HSC activation, and hepatic fibrosis, independent of caspase 3 activation and hepatocellular apoptosis.
Fig. 4. **HSC activation and collagen deposition during diet-induced steatohepatitis are markedly decreased in Casp1−/−.** (A-C) RT-PCR analysis of HSC activation markers transforming growth factor beta (TGFβ), collagen type I alpha 1 (COL1A1), alpha smooth muscle actin (αSMA), cysteine- and glycine-rich protein 2 (CRP2), mRNA expression in liver of Casp1−/− mice compared to WT mice (n = 5-7 in each group). (E) Western blot of αSMA on whole liver lysates in WT and Casp1−/− MCD-fed mice compared to CTL-fed mice and (F) corresponding densitometric analysis to HSC70 (n=3). (G) Collagen fibers were stained with Sirius red and (H) quantified using the surface area stained per 40x field area (n = 5-7 in each group) excluding blood vessels. Results are expressed as mean ± S.E.M. * P < 0.05; ** P < 0.01; ***P < 0.001 compared to controls.
Fig. 5. Protection from MCD-diet induced fibrosis in Casp1⁻/⁻ is independent of caspase 3 activation and hepatocellular apoptosis. (A, B) Representative microphotographs of TUNEL staining, and active caspase 3 immunohistochemistry (40x). (C, D) Hepatocyte apoptosis in liver sections was quantified by counting the number of TUNEL positive cells, and cleaved caspase 3-positive cells in 5 random microscopic fields (40x). Results are expressed as mean ± S.E.M. * P < 0.05; ** P < 0.01 compared to controls.
Selective Kupffer cell depletion in MCD-induced steatohepatitis reduces caspase-1 activation and protects against fibrogenesis and fibrosis. The findings of inflammasome activation and marked increased in caspase-1 and IL-1β mainly from non-parenchymal cells of the liver on MCD-fed animals in conjunction with the fact that the inflammasome is primarily present in monocytes and macrophages led us to the hypothesis that Kupffer cells, the liver resident macrophages, are the main source for these changes. To test this hypothesis we next examine the effects of selective depletion of Kupffer cells on caspase-1 expression and MCD induced liver damage. C57BL/6 WT mice on a 6-week MCD diet were injected twice during the last week of the diet with clodronate (CLOD) or PBS vehicle (PBS) liposomes intravenously. Hepatic steatosis was unchanged between the CLOD and PBS treated MCD-fed mice (Fig. 6A). Hepatic F4/80 immunostaining demonstrated the effectiveness of CLOD treatment in the depletion of Kupffer cells in the liver (Fig 6A). This was further confirmed by measurement of mRNA levels by RT-PCR of TNFα, F4/80 and CD68, which were markedly decreased in the CLOD-treated group compared to PBS-treated MCD-fed mice (Fig 6B). More importantly, caspase-1 protein expression was also significantly reduced in CLOD-treated MCD-fed mice as detected by both immunohistochemistry of liver tissue and immunoblot analysis of whole liver lysates (Fig 6C - E). These changes were associated with a decrease in the expression of genes involved in fibrogenesis (Fig. 6F) as well as a dramatically reduced in collagen deposition in CLOD-treated MCD-fed mice (Fig 6G). These
data strongly suggest that Kupffer cells are the main cellular source of active caspase-1 in MCD-diet induced steatohepatitis.
Fig. 6. Kupffer cell depletion abrogates caspase-1–mediated diet-induced steatohepatitis. (A) Representative photomicrographs of H&E, and F4/80 immunostaining in the liver of PBS (n=7) or CLOD–treated (n=3) MCD-fed mice. (B) RT-PCR analysis of tumor necrosis factor (TNFα), and macrophage markers F4/80 and CD68 expression in the liver of PBS (n=7) or CLOD–treated (n=3) MCD-fed mice. (C) Representative photomicrographs of caspase-1 immunostaining, on liver tissue from the two groups of mice, and (D, E) Western blot analysis of caspase-1 performed on whole liver tissue lysates from PBS (n=7) or CLOD–treated (n=3) MCD-fed mice with densitometric analysis. (F) RT-PCR analysis of fibrogenesis genes αSMA and TGFβ expression in the liver of PBS (n=5) or CLOD–treated (n=3) MCD-fed mice (G) Collagen fibers were stained with Sirius red. Results are expressed as mean ± S.E.M. * P < 0.05 compared to controls.
Fig. 7. Proposed model for role of inflammasome and caspase-1 activation in tissue damage and fibrosis in steatohepatitis. In the process of NASH development, different lipotoxic substances such as cholesterol crystals, or free fatty acids may induce the processing and activation of caspase-1 predominantly in Kupffer cells in the liver, and to a lesser extent in hepatocytes. Hepatic caspase-1 activation induce cleavage of IL-1 into its mature form IL-1beta resulting in increased inflammation and tissue damage including the activation of hepatic stellate cells. This results in fibrogenesis and fibrosis development. Therapy target at inhibiting the inflammasome, or caspase-1 activation may be a novel therapeutic target for treatment of NASH.
**Discussion**

The principal findings of this study relate to the role of caspase-1 during NASH development. The results demonstrate that NASH induced by MCD feeding is associated with caspase-1 activation in the liver, while caspase-1 suppression is associated with dissociation between hepatic triglyceride accumulation and inflammatory activity, and protects against HSC activation and fibrosis development. These effects were independent of caspase-3 activation and hepatocyte cell death. Furthermore, we identified Kupffer cells as a key cellular source of active caspase-1 during MCD diet-induced steatohepatitis.

Since the original description that caspase-3 and -7 activation and TUNEL positive cells are a characteristic pathologic features in the liver of NASH patients [122], a growth of data mainly from experimental studies have suggested that caspase activation, mainly caspase-3 is a key process involved in NASH pathogenesis [123]. As a result, the targeting of caspase activity has gained significant attention for developing of both novel therapeutic as well as diagnostic strategies for NASH patients. A recent pre-clinical study tested a pan-caspase inhibitor VX-166 in an animal model of NASH[131]. Obese leptin receptor deficient db/db mice were fed methionine and choline-deficient (MCD) diet to induce NASH and liver fibrosis. Mice gavaged daily with VX-166 showed a marked reduction in hepatic caspases activity, decreased levels of mature IL-1β and IL-18 in the liver, and liver fibrosis.

Caspases belong to a family of highly conserved cysteine-dependent aspartate-specific acid proteases that use a cysteine residue as the catalytic
nucleophile and share a stringent specificity for cleaving their substrates after aspartic acid residues in target proteins [127]. They are synthesized as inert zymogens and upon receipt of apoptotic stimuli, cells activate initiator caspases such as, caspase-1, 2, 8, 9, and 10 that, in turn, proteolytically cleave and activate effector caspases including caspase 3, 6, and 7 [128]. Caspases have been further categorized as either proinflammatory or proapoptotic, depending upon their participation in these cellular programs. The proinflammatory caspases include caspase-1, 11 and 12 in mouse and caspase-1, 4, and 5 in human. The relative contribution of pro-apoptotic versus pro-inflammatory caspases to liver pathology during NASH development as well as in the protective effects of pan-caspase inhibitors remain incompletely understood. Our results confirmed the findings from Witek and colleagues [131] demonstrating that MCD feeding results in marked caspase-1 activation. Our current data further extend these observations by demonstrating that activated cleaved caspase-1 localized predominately on non-parenchymal sinusoidal cells in the liver, and to a lesser extent to hepatocytes. Suppression of caspase-1 activation resulted in decreased tissue inflammation despite an increase in triglyceride deposition and hepatic steatosis. Moreover, while as expected after six weeks on the MCD diet wild type animals showed a marked increase in the expression of various genes involved in HSC activation and fibrogenesis, these changes were significantly reduced in the Casp1−/− mice. More importantly, wild type animals on the MCD diet but not Casp1−/− mice showed a significant increase in collagen deposition. These changes were independent of hepatocyte caspase-3 activation, hepatocellular injury as evidence
by ballooning degeneration of hepatocytes and elevation of serum transaminases, and cell death which occurred to a similar extent in both WT animals and Casp1−/− mice on the MCD diet. The precise mechanisms resulting in the catalytic processing of pro-caspase-1 into its enzymatically active form during NASH development will require further investigation but multiple processes that are known to induce the assembly of the inflammasome, the caspase-1-activating complex, such as increase in reactive oxygen species production, and lysosomal permabilization and release of cathepsins into the cytosol are known to be present in both experimental model of NASH as well as humans with this condition [125,132].

Our key finding of a mixed pattern of cellular expression of caspase-1 in this model of steatohepatitis, in conjunction with the recent report that fatty acids may induce activation of caspase-1 in isolated hepatocytes [133] led us to further investigate the potential cellular source of this protease. Interestingly, we observed that selective depletion of Kupffer cells by clodronate treatment markedly reduced the protein expression of caspase-1 in the liver of MCD-fed animals as well as significantly reduced the amount of collagen deposition pro-inflammatory cytokine TNFα in a similar manner to the Casp1−/− mice on the MCD diet. These results strongly suggest that Kupffer cells are a key cellular source of active caspase-1 in MCD-induced steatohepatitis, which plays an important role in the pathogenesis of this model through inflammation and hepatic stellate cell activation and fibrogenesis. Future studies such as those using cell-type specific caspase-1 knockout mice will be required to further dissect the role
of caspase-1 activation in other cell types in the liver in vivo and their potential
distinct role in the pathophysiologic changes observed during NASH
development.

In summary, the current studies uncover the role of hepatic caspase-1 activation in experimental NASH. The results support a model in which during the development of NASH, caspase-1 activation in Kupffer cells results in induction of pro-inflammatory signaling and hepatic stellate cell activation which are then responsible for collagen deposition and fibrosis (Fig. 6). These data provide new insights into the pathogenesis of liver damage in NASH, and identify potential novel molecular targets for therapeutic intervention in this highly common and potentially serious disease.
CHAPTER 4

High-fat diet Induces Caspase-1 -Dependent Markers of Early Fibrosis in Mice

Portions of this chapter are under review at PLOS One

Summary

Nonalcoholic steatohepatitis (NASH) is associated with caspase activation. However, a role for pro-inflammatory caspases or inflammasomes has not been explored in diet-induced liver injury. Our aims were to examine the role of caspase-1 in high fat-induced NASH. C57BL/6 wild-type and caspase 1-knockout (Casp1−/−) mice were placed on a 12-week high fat diet. Wild-type mice on the high fat diet increased hepatic expression of pro-caspase-1 and IL-1β. Both wild-type and Casp1−/− mice on the high fat diet gained more weight than mice on a control diet. Casp1−/− mice had greater adiposity on the high fat diet than wild-type. Hepatic steatosis and TG levels were increased in wild-type mice on high fat diet, but were attenuated in the absence of caspase-1. ALT levels were elevated in both wild-type and Casp1−/− mice on high fat diet compared to control. Hepatic Tnfα and Mcp-1 mRNA expression was increased in wild-type mice on high fat diet, but not in Casp1−/− mice on high fat diet. αSMA positive cells, Sirius red staining, and Col1α1 mRNA were increased in wild-type mice on high fat diet compared to control. Deficiency of caspase-1 prevented those increases. In summary, the absence of caspase-1 ameliorates the injurious effects of high fat diet-induced obesity on the liver. Specifically, mice deficient in caspase-1 are
protected from high fat-induced hepatic steatosis, inflammation and early fibrogenesis. These data point to the inflammasome as an important therapeutic target for NASH.

**Introduction**

Consumption of high-energy diets and weight gain are associated with development of several metabolic complications such as insulin resistance and hepatic steatosis, characteristics of an early stage within the spectrum of Non-Alcoholic Fatty Liver Disease (NALFD). NAFLD is a common form of chronic liver disease, affecting both adults and children, which is strongly associated with obesity and insulin resistance [14,15]. 10-20% of adults have hepatic steatosis, which is characterized by triglyceride accumulation in liver cells and follows a benign non-progressive clinical course [116,117]. Nonalcoholic steatohepatitis (NASH) is defined as lipid accumulation with evidence of cellular damage, inflammation and early to moderate fibrosis [118]. NASH is a serious condition, as approximately 3-15% of these patients progress to cirrhosis, with complications including portal hypertension, liver failure and hepatocellular carcinoma [119,120,121]. In particular, the mechanisms responsible for liver injury and disease progression remain incompletely understood, but are of significant biomedical importance, as identification of cellular and molecular processes of NASH pathogenesis may help to identify novel diagnostic and therapeutic targets for this highly prevalent and potentially serious disease.
Until recently, progression of disease from NAFLD to NASH was modeled as a two-hit process. The first hit is proposed to be the accumulation of lipids within hepatocytes, and considered a requirement for later events leading to liver injury [28,134]. Lipid accumulation occurs as a result of availability of excess free fatty acids (FA) coming from high-energy/high fat diets or released from adipose tissue, as well as an increase in hepatic fatty acid synthesis and decrease in degradation [31]. The second hit is proposed to be a multifactorial process likely involving a combination of apoptosis and necrosis, oxidative stress and lipid peroxidation, proinflammatory cytokine and chemokine production, dysregulated adipokine expression and mitochondrial dysfunction [135]. All of these insults cumulatively contribute to the progression of NAFLD to NASH and fibrosis. Despite this characterization of multiple contributors to disease progression, there remains a dearth of evidence to explain the molecular mechanisms of how these proposed "second hits" in diet-induced obesity result in the progression from steatosis to NASH.

The inflammasome is an innate immune response that involves the formation of a multiprotein caspase-1-activating complex [80]. The inflammasome complex contains cysteine-aspartate protease-1 (caspase-1), Apoptosis-associated speck-like protein containing a CARD (ASC), and a Nod-like receptor (NOD) protein (NLRP). The inflammasome senses danger signals through intracellular NLRPs. Among known inflammasomes (NLRP1, NLRP3, NLRC4, AIM, and IPAF), NLRP3 is responsible for the activation of immune cells in response to tissue injury and cell death via monosodium urate [136].
Formation of the inflammasome complex leads to the activation of caspase-1, which in turn proteolytically cleaves interleukin-1 (IL-1) and -18 into their mature active forms [137].

Multiple lines of evidence suggest a role for the inflammasome in NASH, for example, IL-1 receptor (IL-1R) is thought to play a role in NASH since IL-1 knockout and IL-1R knockout mice are protected from methionine and choline deficient (MCD) diet-induced steatohepatitis [4,5,102,138]. We and others have shown an increase in expression and activation of caspase-1 in the MCD mouse model of NASH [102,138,139]. In addition, pharmacological inhibition of all caspases blocks hepatocyte apoptosis and the progression of MCD-induced fibrosis in mice, but does not improve hepatocellular injury assessed by plasma alanine aminotransferase (ALT), nor hepatocellular ballooning, steatosis or inflammation [140,141]). While pointing to caspases as important mediators of molecular mechanisms in NASH, these data were limited to the MCD diet, which can be considered a non-pathogenic NASH model. The MCD diet induces the phenotypic changes of NASH in the liver, but does not reflect the dietary etiology, pathogenesis, or disease mechanisms seen in NASH patients [104,142]. In addition, the MCD diet causes non-characteristic features of NASH, such as weight loss and wasting syndrome [143].

High energy/high fat diets and obesity lead to a chronic state of low-grade inflammation, hyperinsulinemia, hyperglycemia and increased ROS production and cell death. We tested the hypothesis that caspase-1 plays a role in the pathogenesis of high fat diet-induced liver injury. In order to test this hypothesis,
we exposed caspase-1 deficient mice (Casp1−/−) to a high fat diet for 12 weeks. The absence of caspase-1 decreased hepatic steatosis and indicators of the capacity for FA synthesis. Casp1−/− mice were protected from high fat diet-induced hepatic inflammatory cytokine expression. In addition, absence of caspase-1 protected mice from early stages of fibrogenesis, suggesting that caspase-1 is necessary for an initial pro-fibrotic response during obesity-induced liver injury.

Results

High fat diet increases the expression of caspase-1 and IL-1β in the liver.

C57BL/6 mice allowed free access to a high fat diet had increased hepatic expression of immunoreactive caspase-1 compared to mice on the control diet (Fig. 8a, b). Further, western blot analysis revealed that high fat diets also increased the quantity of mature IL-1β in the liver compared to mice of the control diet (Fig. 8a, c). Wild-type mice on the high fat diet also had increased expression of IL-1 mRNA expression in the liver; this increase was associated with increased total IL-1 protein in the liver (Fig. 8d, e.)
Figure 8. Caspase-1 and IL-1β expression is increased after high fat feeding. Livers of wild-type mice on the control or high fat diet were analyzed for pro-caspase-1 and IL-1β expression by immunoblot (A). Densitometric analysis was done for each protein normalized to HSC70 (B and C). mRNA for IL-1 expression in the liver of mice on the control or high fat diet was analyzed by RT-PCR (D). IL-1 was assessed by ELISA in whole liver tissue (E). Values with different superscripts are significantly different from one another ($p < 0.05$). n=4 control, n=6 high fat.
**Caspase-1 knockout mice develop obesity and increased adiposity on a high fat diet.** If caspase-1 contributes to high fat induced liver injury, then caspase-1 knockout mice (Casp1⁻/⁻) on a high fat diet should be protected from high fat diet-induced liver injury. Wild-type and Casp1⁻/⁻ mice on high fat diets consumed similar amount of food and gained more body weight than control-fed mice, but Casp1⁻/⁻ mice diet gained less than wild-type mice on the high fat diet (Table1). Wild-type and Casp1⁻/⁻ mice on both control and high fat diets had similar liver weight (Table 2). High fat diets increased the adiposity wild-type mice, measured by small animal magnetic resonance imaging (Fig. 9a). While both wild-type and Casp1⁻/⁻ mice on the high fat diet increased subcutaneous, visceral and total body adipose tissue volume compared to the control diet (Fig. 9b). High fat diets increased subcutaneous and total body adipose volume to a greater extent in Casp1⁻/⁻ mice compared to wild-type mice (Fig. 9b).
Table 2. Absence of caspase-1 protects from high fat-induced obesity
Wild-type (C57BL/6) and Casp1<sup>−/−</sup> mice were maintained on either a control or high fat diet for 12 weeks. Body weight at week 0 and 12 were measured. Food intake was measured weekly. Liver weight was measured at 12 weeks. Values represent means ± SEM. Values with different superscripts are significantly different from one another (<i>p</i>&lt;0.05). n=4 control, n=6 high fat.

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6 Control</th>
<th>C57BL/6 High fat</th>
<th>Casp1&lt;sup&gt;−/−&lt;/sup&gt; Control</th>
<th>Casp1&lt;sup&gt;−/−&lt;/sup&gt; High fat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Body Weight (g)</td>
<td>26.5±1.2</td>
<td>27.7±0.9</td>
<td>29.7±1.4</td>
<td>29.1±0.7</td>
</tr>
<tr>
<td>Final Body Weight (g)</td>
<td>29.1±1.4&lt;sub&gt;a&lt;/sub&gt;</td>
<td>39.2±1.6&lt;sub&gt;b&lt;/sub&gt;</td>
<td>35.0±2.3&lt;sub&gt;b&lt;/sub&gt;</td>
<td>36.4±1.2&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
<tr>
<td>Body Weight Change (g)</td>
<td>2.6±0.8&lt;sub&gt;a&lt;/sub&gt;</td>
<td>11.5±1.0&lt;sub&gt;b&lt;/sub&gt;</td>
<td>5.4±1.0&lt;sub&gt;a&lt;/sub&gt;</td>
<td>7.3±0.6&lt;sub&gt;c&lt;/sub&gt;</td>
</tr>
<tr>
<td>Food Intake (g/day/animal)</td>
<td>1.5±0.1&lt;sub&gt;a&lt;/sub&gt;</td>
<td>1.5±0.03&lt;sub&gt;a&lt;/sub&gt;</td>
<td>1.9±0.1&lt;sub&gt;b&lt;/sub&gt;</td>
<td>1.5±0.1&lt;sub&gt;a&lt;/sub&gt;</td>
</tr>
<tr>
<td>Liver Weight (g)</td>
<td>1.5±0.3</td>
<td>1.7±0.2</td>
<td>1.5±0.2</td>
<td>1.9±1.1</td>
</tr>
</tbody>
</table>
Figure 9. Adiposity is increased in caspase-1 knockout mice. Mice on the control and high fat diets were analyzed for body adiposity. Fat content of subcutaneous and visceral depots were calculated from three images (B). Total adipose volume was calculated from subcutaneous and visceral depots (B). Values represent means ± SEM. Values with different superscripts are significantly different from one another ($p < 0.05$). $n=5$ C57BL/6, n=4 Casp1$^{-/-}$. 
The absence of caspase-1 prevents high fat diet-induced hepatic steatosis.

Wild-type mice fed a high fat diet had increased hepatic steatosis as assessed by H&E staining (Fig. 10a, Table 3). This response was reduced in the absence of caspase-1 (Fig. 10a). Histopathological analysis revealed the NAFLD Activity Score was greater in wild-type mice on the high fat diet than Casp1−/− mice on the high fat diet (Fig. 10b). Biochemical analysis revealed an increase in hepatic triglyceride wild-type mice fed high fat diet compared to control, but not in Casp1−/− mice on high fat diet (Fig. 10c). Activity of plasma alanine aminotransferase (ALT), an indicator of hepatocyte injury, was elevated with high fat feeding; this increase was independent of genotype (Fig. 10d).
Figure 10. Caspase-1 knockout mice are protected from high fat-induced hepatic steatosis. Representative images of H&E stained livers in wild-type and Casp1−/- mice on control or high fat diet (10x) (A). NAFLD activity score of wild-type and Casp1−/- mice on the high fat diet was assessed by histopathology of H&E stained livers in a blinded-fashion by BPG (B). Hepatic triglyceride (TG) levels were measured biochemically from mice on the control or high fat diets (C). Plasma alanine aminotransferase (ALT) levels were analyzed from mice on the control or high fat diets (D). Values represent means ± SEM. Values with different superscripts are significantly different from one another (p < 0.05). n=4 control, n=6 high fat.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6 Control</th>
<th>C57BL/6 High fat</th>
<th>Casp1&lt;sup&gt;-/-&lt;/sup&gt; Control</th>
<th>Casp1&lt;sup&gt;-/-&lt;/sup&gt; High fat</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steatosis</strong></td>
<td>0±0</td>
<td>2±0.3</td>
<td>0±0</td>
<td>1±0.3</td>
</tr>
<tr>
<td><strong>Ballooning</strong></td>
<td>0±0</td>
<td>1±0.2</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td>0±0</td>
<td>1±0.2</td>
<td>0±0</td>
<td>0±0</td>
</tr>
</tbody>
</table>

Pathologist: BPG. Data are represented as Mean ± SEM.

Table 3. **Histopathological analysis of mice on the high fat diet.** H&E slides of the livers of mice on the control and high fat diets were analyzed for steatosis, inflammation, and ballooning. H&E sections were scored in a blinded fashion by BPG. Values represent means ± SEM. n=4 control, n=6 high fat.
Expression of mRNA for sterol receptor element binding protein-1c (Srebp1c) key transcription factor regulating lipid synthesis, was increased with high fat feeding in wild-type mice, but not in *Casp1*/*mice (Fig. 11a). Similarly, expression of mRNA for stearoyl-CoA desaturase–1 (Scd-1), a downstream target of sterol receptor element binding protein-1c, was increased in wild-type mice on a high fat diet (Fig. 11b). The absence of caspase-1 ameliorated this response (Fig. 11b). Since IL-1β contributes to hepatic steatosis via suppression of peroxisome proliferator-activated receptor-alpha (Pparα) activity [144,145,146], we examined Pparα expression in wild-type and *Casp1*/* after a high fat diet. Expression of mRNA for Pparα was not affected by diet in either genotypes; however was decreased in *Casp1*/* compared to wild-type (Fig. 11c).
Figure 11. High fat-induced expression of lipogenesis-related genes is attenuated in caspase-1 knockout mice. mRNA expression of SREBP-1c (A), SCD-1 (B) and PPARα (C) in the liver of wild-type compared to Casp1−/− mice on control or high fat diets. Values represent means ± SEM. Values with different superscripts are significantly different from one another (p < 0.05). n=4 control, n=6 high fat.
Caspase-1 knockout mice are protected from high fat diet-induced markers of inflammation. Since caspase-1 is a pro-inflammatory protease, its contribution to high fat–induced hepatic inflammation was investigated. Histopathological analysis of H&E stained liver sections displays inflammation in wild-type mice on the high fat diet compared to control diet (Table 3). Expression of mRNA for tumor necrosis factor alpha (Tnfα) and monocyte chemotactic protein-1 (Mcp-1) was increased with in wild-type mice on the high fat diet (Fig. 12a, b). Expression of these inflammatory markers was not increased in Casp1−/− mice on high fat diet (Fig. 12c). Expression of mRNA for pan-macrophage marker F4/80 was unchanged from control in both wild-type and Casp1−/− mice on the high fat diet (Fig. 12c).
Figure 12. High fat-induced expression of inflammatory cytokines and chemokines is attenuated in caspase-1 knockout mice. Inflammatory cytokine/chemokine production in the liver is associated with the early stages of NASH. mRNA expression for Tnfα (A), Mcp-1 (B) and F4/80 (C) in the liver of wild-type mice on control or high fat diets was performed by RT-PCR. Values represent means ± SEM. Values with different superscripts are significantly different from one another \((p < 0.05)\). n=4 control, n=6 high fat.
Early indicators of fibrogenesis are induced by high fat diet in wild-type, but not in Caspase-1 knockout mice. Given the recent findings of the inflammasome and caspase-1 in liver fibrosis [138,139,140], we evaluated the role of caspase-1 in high fat diet-induced early fibrogenesis. Expression of the hepatic stellate cell (HSC) activation marker alpha-smooth muscle actin (αSMA) by immunohistochemistry was increased in the perisinusoidal space of the livers of wild-type mice on the high fat diet compared to control, but not in Casp1<sup>-/-</sup> mice (Fig. 13a, c). Sirius Red staining, an indicator of extracellular matrix deposition, was greater in wild-type mice on the high fat diet compared to control diet and was present in a chicken-wire manner (Fig. 13b, d). However, in the absence of caspase-1, a high fat diet did not induce extracellular matrix deposition over mice on the control diet (Fig. 13b and d). Consistent with this finding, mRNA expression for collagen type 1-alpha-1 (COL1α1) was increased in the sinusoidal space in wild-type mice on a high fat diet; but was not increased Casp1<sup>-/-</sup> mice (Fig. 13e).
Figure 13. **High fat-induced early fibrogenesis is prevented in caspase-1 knockout mice.** Early to moderate fibrosis in the liver is associated with high fat diet feeding. αSMA immunohistochemistry (A, C), Sirius Red staining (B, D) and Col1α1 mRNA (E) in the liver of mice on the control or high fat diets. Values represent means ± SEM. Values with different superscripts are significantly different from one another (p < 0.05). n=4 control, n=6 high fat.
Discussion

The progression of NAFLD and NASH involves the development of hepatic steatosis, increased expression of inflammatory cytokines and chemokines, and the eventual deposition of extracellular matrix proteins. This process involves complex interactions between multiple cell types within the hepatic architecture. The contribution of cells of the innate immune system and HSCs are particularly critical to the progression from NAFLD to NASH. The purpose of this study was to test the hypothesis that caspase-1 plays a role in the pathogenesis of high fat diet-induced NASH. Caspase-1 and IL-1β expression were increased in mice on the high fat diet. While both wild-type and Casp1−/− mice became obese on the high fat diet, Casp1−/− mice were protected from a number of the phenotypic characteristics of early NASH. For example, the high fat diet increased hepatic steatosis and expression of lipogenesis related genes in wild-type mice, but Casp1−/− mice were protected from these diet-induced phenotypes. These data indicate that caspase-1 directly and/or indirectly influences hepatocytes in the development of fatty liver. Further, while the high fat diet increased inflammatory cytokine and chemokine expression in wild-type mice, this response was attenuated in Casp1−/− mice, suggesting a role for caspase-1 in macrophages during the progression to NASH. Finally, our data also indicate that caspase-1 plays an important role at early stages of fibrogenesis in response to high fat diets. Evidence for activation of HSCs and accumulation of extracellular matrix proteins was apparent in wild-type mice after 12 weeks of high fat diet. Importantly, Casp1−/− mice had decreased fibrogenesis compared to
wild-type in response to a high fat diet, suggesting either a direct and/or indirect role of caspase-1 in the activation of HSCs. 

*Casp1−/−* mice fed a high fat diet exhibited greater adiposity than wild-type, associated with reduced accumulation of liver triglycerides during the high fat diet feeding. The reduced accumulation of hepatic triglycerides in the *Casp1−/−* mice might also be due to decreased hepatic lipogenesis, since the *Casp1−/−* mice expressed less mRNA for two genes involved in hepatic lipogenesis, SREBP-1c and its downstream target SCD-1. In contrast, lower hepatic triglyceride is not likely due to increased fatty acid oxidation, as expression of Pparα mRNA was reduced in *Casp1−/−* mice, indicative of a reduced capacity for fat oxidation. This is in contrast with findings from Steinstra et al. that demonstrate *Casp1−/−* mice have increased energy expenditure [101]. The discrepancy may lie with the length of feeding or simply because the energy expenditure does not account for liver fat oxidation. The livers of *Casp1−/−* mice on the high fat diet have less triglyceride to oxidize. Thus, genes for oxidation are suppressed. Another way in which caspase-1 may contribute to steatosis is via regulation of cytokine and chemokine expression in the liver. Tnfa promotes the maturation of SREBP-1 [147] and Mcp-1 may directly regulate hepatic lipid homeostasis, independent of its chemokine activity [148]. Thus, lower expression of hepatic Tnfa and Mcp-1 in *Casp1−/−* mice compared to wild-type mice on the high fat diet likely contributed to a decrease in high fat diet-induced hepatic steatosis.

The expression of caspase-1 and other components of the inflammasome is constitutive in monocytes. Activation of the inflammasome in monocytes is
therefore primarily controlled by recruitment of proteins to the inflammasome complex. In contrast, expression of inflammasome components and caspase-1 can be induced in non-myeloid cells, such as hepatocytes or other non-parenchymal cells [149]. Thus, the inflammasome and caspase-1 is differentially regulated in multiple cell types of the liver; activity in each cell type may make unique contributions to hepatic pathophysiology during disease progression.

Caspase-1 has been localized to both hepatocytes and non-parenchymal cells of the liver after MCD feeding and contributes to inflammation and fibrogenesis in this model of NASH [138]. However, very little further data is available to explain the cell-type specific mechanisms responsible for promoting caspase-1 activity and inflammasome formation in NASH.

The role of LPS activation of TLR-4 on hepatic Kupffer cells, leading to expression of pro-inflammatory cytokines and chemokines, during high fat diet induced liver injury is well studied. Importantly, TLR4/-/- mice are protected from high fat diet induced liver injury [44]. TLR ligands can include both pathogen-associated molecular patterns (PAMPs) (eg. lipopolysaccharide (LPS)) and damage-associated molecular patterns (DAMPs) (eg. free fatty acids (FFA). High fat diet feeding not only increases the availability of FFA, but also increases plasma LPS to a concentration equivalent to that seen in low-level metabolic endotoxemia and bacterial overgrowth [150]. This metabolic endotoxemia is considered a key inducer of metabolic syndrome [150], with multiple cytokines (Tnfα and IL-1β) and chemokines (e.g. Mcp-1) contributing to the progression of both alcoholic-liver disease (ALD) and NAFLD [148,151,152,153].
Ganz et al. recently reported that LPS, a ligand for TLR4, may be an important regulator of hepatic expression of caspase-1 and the NLRP3 inflammasome [139]. These data provide an important potential link between the inflammasome and TLR-4-dependent responses. Our data are consistent with the hypothesis that caspase-1 contributes to inflammatory cytokine and chemokine expression in the liver during high fat diet feeding. When wild-type mice were fed the high fat diet for 12 weeks, expression of hepatic Tnf-α and Mcp-1 mRNA were increased; this response was attenuated in Casp<sup>−/−</sup> mice. This difference might be due to differential recruitment of monocytes/macrophages or differences in macrophage activation between wild-type and caspase-deficient mice. Here we find that high fat diet-induced increases in expression of monocyte/macrophage marker F4/80 was not affected by genotype, suggesting that caspase-1 is important for regulating the sensitivity of Kupffer cells to activation during high fat diet-induced obesity, rather than recruitment and/or proliferation of Kupffer cells in the liver.

In addition to a role in steatosis and inflammatory cytokine activity, caspase-1 also contributed to early stages of hepatic fibrosis in high fat diet-induced obesity. Fibrogenesis in the liver requires the activation of hepatic stellate cells, characterized by an increase in expression of αSMA expression. αSMA-positive cells were increased in the livers of wild-type mice on the high fat diet, but were reduced in the parenchyma of Casp<sup>−/−</sup> mice. Wild-type mice on the high fat diet also increased expression of Col1α1 and a deposition of chicken-wire-like extracellular matrix in the perisinusoidal space, indicative of early phases of
fibrogenesis. Casp1−/− mice were protected from these markers of early fibrogenesis in response to a high fat diet. These data suggest that caspase-1 is important to the activation of HSC and promotes high fat diet–induced early fibrogenesis.

These data add to the increasing evidence for an innate immune function of HSCs. For example, TLRs are expressed by HSC [154] and have been associated with the progression of fibrosis. TLR4−/− mice are protected from bile duct ligation, thioacetamide (TAA) or carbon tetrachloride (CCL4)–induced fibrosis [155]. Further, inflammasome components are expressed by both LX-2 cells and primary cultures of HSC, regulating HSC function in culture and in vivo during experimental liver fibrosis induced by CCL4 or TAA [136]. Mice lacking inflammasome components ASC and NLRP3 have reduced thioacetamide or CCL4-induced liver fibrosis [136]. However, the cellular and molecular mechanisms for caspase-1–dependent HSC activation and fibrogenesis remain to be elucidated and will require further investigation.

Taken together, the present data suggest that caspase-1 is expressed and functional in multiple cell types of the liver at different times of high fat diet-induced NASH, promoting steatosis, inflammatory cytokine production and the activation of HSC leading to early fibrogenesis. This data is consistent with the concept that the inflammasome is differentially regulated in monocytic and non-monocytic cells. Caspase-1 plays a role in protecting non-monocytic cells, such as hepatocytes, from steatosis [102], whereas it contributes to the production of inflammatory cytokines in monocytic cells, like Kupffer cells. Data from both
high fat diet (presented here) and MCD diet-induced [138] liver injury clearly indicates that caspase-1 also contributes to the regulation of HSC function in different mouse models of NASH, indicating a need for further studies investigating the regulation and role of the caspase-1 inflammasome in hepatic stellate cells.
CHAPTER 5

Discussion and Future Directions

The preceding chapters have investigated the role of caspase-1 activation in diet-induced murine NASH. The MCD diet model of murine NASH induced the expression and activation of hepatic caspase-1, leading to the production of IL-1β. Caspase-1 expression was localized to both hepatocytes and non-parenchymal cells. Isolated non-parenchymal cells had higher expression of caspase-1 and IL-1β than isolated hepatocytes. The eradication of Kupffer cells from mice on the MCD diet with clodronate liposomes, the inflammatory and fibrogenesis phenotypes recapitulated that of \( \text{Casp}1^{-/-} \) mice on the MCD diet. Therefore, we demonstrated that Kupffer cells are a key source of caspase-1 in MCD-induced NASH. The high-fat diet model of murine NASH also induced the expression and activation of caspase-1 and the processing of IL-1β. \( \text{Casp}1^{-/-} \) mice on the MCD diet were protected from MCD-induced NASH, specifically from inflammatory activity and HSC activation and fibrogenesis. These effects were independent of hepatic TG accumulation and hepatocellular apoptosis. The high-fat diet also induced hepatic expression of caspase-1 and IL-1β. \( \text{Casp}1^{-/-} \) mice on the high-fat diet were protected from high-fat diet-induced obesity, while their adiposity was increased over wild-type mice on the high-fat diet. \( \text{Casp}1^{-/-} \) were also protected from a number of phenotypic characteristics of early NASH, including hepatic steatosis and TG accumulation, expression of lipogenesis genes. These data demonstrate a role for caspase-1 in hepatic steatosis. Furthermore, \( \text{Casp}1^{-/-} \) mice
were protected from the expression of inflammatory genes, suggesting a role for caspase-1 in Kupffer cells. These data correspond to the inflammatory phenotype of Casp1−/− mice on the MCD diet. Finally, our data show that caspase-1 is playing a role in the activation and progression of early high-fat diet–induced fibrogenesis. Casp1−/− mice on the high-fat diet did not have increases in numbers of αSMA positive cells, extracellular matrix deposition and expression of collagen compared to wild-type mice on the high-fat diet. These data point to a role for caspase-1 in HSC activation and fibrogenesis. However, there still remains many questions related to the mechanisms of caspase-1–mediated steatohepatitis.

I. What are the mechanisms responsible for activating caspase-1 in NASH?

Since there are many activators of caspase-1, it would be exciting to discover which of these plays a role in caspase-1 activation in NASH. Some of the obvious activators would be LPS and FFA, as some investigators have shown these compounds to be activators of hepatocyte caspase-1 [102,139]. ROS is implicative in inflammasome activation [156,157,158]. Intracellular K+, frustrated phagocytosis and phagolysosomal release of cathepsin B all converge upon ROS generation. Schroder et al. and Zhao et al. shows that the NLRP3 inflammasome is activated in pancreatic islet cells during insulin resistance and hyperglycemia [159], and is mediated by an interaction between thioredoxin and thioredoxin interacting protein [160], resulting in NLRP3 assembly and caspase-1 activation.
Similarities between NASH and alcoholic liver disease suggest commonality in pathogenic mechanisms. Petrasek et al. recently discovered that caspase-1 and IL-1β are increased upon alcohol feeding and are inhibited by IL-1Ra. They also demonstrate protection of Casp1−/−, IL-1R−/− and ASC−/− mice from alcohol-induced steatohepatitis [161]. These results confirm the role of the inflammasome in steatohepatitis, and point to alcohol as an activator of caspase-1.

Gut-Liver Axis

Recently there has been an increase in attention towards gut microbiota and liver disease [162]. Composition of intestinal microbes have been correlated with NASH injury[163]. Henao-Mejia et al. investigated the role of the inflammasome on gut microbiota dynamics in short-term MCD-induced NASH [164]. They concluded that mice lacking inflammasome components had alterations in gut microbiota that contribute to exacerbated NASH injury. This data is directly contradictory to our data that indicates the inflammasome is causative to NASH pathogenesis. The discrepancy may lie within the length of the diets, as well as the difference in Casp1−/− mice (ours being a caspase-1/11 double knockout. More studies are needed to further investigate the contribution of gut microbiota in NASH in general, in addition to its effect on inflammasome-mediated liver injury.

The role of NASH models

Discrepancies in data from Chapter 3 and 4, such as the protection of Casp1−/− mice from hepatic steatosis may lie within the etiology and pathophysiology of the models. While the MCD diet provides a useful model for causing TG
accumulation, inflammation, and fibrogenesis, its main mechanisms of action involve depriving the cells from methionine and choline metabolism. As discussed in Chapter 4, there are other problems associated with the MCD diet, including weight loss, which is not seen in patients with NASH. Therefore, the MCD diet bypasses the etiology of obesity and insulin resistance – induced NAFLD/NASH. The high-fat diet model is more pathophysiological because it causes the overloading of FFA directly from diet and provides the first hit in NASH via insulin resistance. As discussed in the introduction, insulin resistance has profound effects on NAFLD progression to NASH.

While we saw early fibrogenesis in duration of the high-fat diet, it is not a typical fibrosis model. Watanabe et al. demonstrates that Nlrap3<sup>−/−</sup> and ASC<sup>−/−</sup> mice are protected from an 8-week high-dose CCl<sub>4</sub> treatment [136]. Therefore, we placed wild-type and Casp1<sup>−/−</sup> mice on two different CCl<sub>4</sub> models of fibrosis. The 72-hour acute model induces rapid indicators of fibrosis, such as αSMA and collagen. The 5-week, or chronic model induces indicators of prolonged and proliferative fibrosis, such as expression of extracellular matrix. If the results indicate that Casp1<sup>−/−</sup> mice are protected from fibrosis in either model, it suggests a role for caspase-1 in chemical-induced fibrosis and toxicity. However, the results of both models indicate no protection from CCl<sub>4</sub> – induced fibrosis (Fig. 14). This either points towards insulin resistance as a critical instigator of caspase-1 in NAFLD/NASH or CCl<sub>4</sub> is too hepatotoxic to target only caspase-1 in our models.
Figure 14. Caspase-1 knockout mice are not protected from carbon tetrachloride–induced fibrogenesis. mRNA expression for αSMA and COL1α1 in livers of wild-type and Casp1−/− mice on a 72 hour CCl4 treatment (A, B) and 5-week chronic CCl4 treatment (C, D).
We can turn to a model of hepatotoxicity in acetaminophen-induced liver injury. Imeada et al. have shown that acetaminophen-induced hepatotoxicity is dependent on sinusoidal endothelial cell TLR9 and the Nlrp3 inflammasome [165]. In this model, TLR9 senses extracellular DNA from damaged hepatocytes. Williams et al. demonstrates that acetaminophen injury had no effect on DAMP release nor neutrophil recruitment and therefore the inflammasome was not a promising target for therapeutics [166]. While it seems hepatotoxicity is still controversial, perhaps lipotoxicity plays a role in caspase-1–mediated NASH. Lipotoxicity is a term to describe non-adipose cell dysfunction and death resulting from toxic mediators, such as FFA, ceramides and free cholesterol. The Nlrp3 inflammasome in adipose tissue and adipose macrophages senses lipotoxic ceramide [100]. However, there have not been many studies exploring caspase-1 in lipotoxicity directly in the liver. Only Csak et al. has treated hepatocytes with saturated fatty acid and shown inflammasome activation, which may confer activation of macrophages as well [102]. Therefore, there is a need to distinguish model systems when we are investigating mechanisms of caspase-1 inflammasome in NAFLD and NASH. The high fat diet is useful for dissecting mechanisms that result from the etiology and pathophysiology of insulin resistance and obesity in both adipose and liver tissues. The MCD diet model is useful for only NASH disease mechanisms in liver, such as inflammation, ER stress, and oxidative stress that result in fibrogenesis. CCl₄ models are useful for hepatotoxic and pro-fibrogenic mechanisms related to NASH.
II. What specific cellular and molecular effects does caspase-1 have in NASH injury?

The previous chapters explored the role of caspase-1 in NASH. The differential expression, activation and regulation of caspase-1 in different cell types of the liver make it difficult to study specific cell-specific roles of caspase-1. In addition to the following discussion, an important set of experiments would be the use of cell-specific caspase-1 knockouts in NASH diet models.

*Hepatocytes*

We have seen the expression and activation of caspase-1 in hepatocytes. Caspase-1 affects the ability of hepatocytes to maintain lipid homeostasis. Chapter 3 discussed that *Casp1*−/− mice were not protected from MCD-induced steatosis, while Chapter 4 discussed the protection of *Casp1*−/− mice from high-fat diet–induced steatosis. Regardless of this discrepancy, caspase-1 plays a role in hepatocytes during NASH liver injury. This may be an early response to an overload of FFA from the circulation.

*Kupffer Cells*

We have also seen a significant role for caspase-1 in Kupffer cells in diet-induced NASH. Chapter 3 investigated caspase-1 in MCD-induced inflammation. We found that *Casp1*−/− mice on the MCD diet were protected from both inflammation as assessed by a histopathological score and the production of inflammatory cytokines. Furthermore, deletion of KCs from the liver of MCD-fed mice demonstrate a protection from inflammation similar to that of *Casp1*−/− mice on the MCD diet. Chapter 4 discussed that *Casp1*−/− mice on the high-fat diet are
protected from the expression of inflammatory genes. Since caspase-1 is known to play a role in monocytes/macrophages, further studies are needed to dissect whether caspase-1 is activating resident KCs or resulting in infiltration of more inflammatory cells from the circulation. Chapter 3 discussed *Casp1*−/− mice on the MCD diet were protected from increases in mRNA for F4/80, a macrophage marker, suggesting a role for caspase-1 in either activation or recruitment of infiltrating macrophages. However, Chapter 4 discussed that *Casp1*−/− mice were not protected from high-fat diet increased expression of F4/80, suggesting a role of caspase-1 in KC activation. An experiment to help answer this question would be a bone marrow transplant procedure. Bone marrow from *Casp1*−/− mice would be eradicated and replaced with wild-type bone marrow. Then these bone marrow chimeras would be placed on the MCD or high-fat diet to see if hepatic inflammation is restored. If it is restored, it suggests that the inflammation is coming from infiltrating cells from the bone marrow or that caspase-1 in other liver cells is responsible for the chemotaxis of these infiltrating cells to sites of liver injury. Other indicators of infiltrating bone marrow cells would be the expression of markers of inflammatory cells, such as Ly6C.

**HSC**

Results from Chapters 3 and 4 suggest a role for caspase-1 in fibrogenesis. Since HSC activation can result from many different stimuli during NASH pathogenesis, the question still remains how caspase-1 is affecting this process. Is caspase-1 acting indirectly through other cell-mediated mechanisms, or is caspase-1 acting directly on or in HSCs? For example, dead or dying hepatocytes
can be phagocytosed by HSC, resulting in their activation and fibrogenesis phenotype. Thus, caspase-1 acting in hepatocytes may cause secondary HSC activation. On the other hand, KC-mediated HSC activation is a well-known mechanism, making KC caspase-1 a source of HSC activation as well. A way in which caspase-1 plays a role in HSC is that of fibrogenesis vs. resolution. Data presented in the preceeding chapters discussed the role of caspase-1 in fibrogenesis and HSC activation. An attractive area of investigation would be the dual role of HSCs in fibrogenesis and HSC cell death. The role of caspase-1 inflammasome in pyroptosis would suggest a mechanism of HSC cell death, both a mechanism of fibrogenesis and resolution. It is conceivable to explore the mechanisms responsible for regulating caspase-1 in fibrogenesis by comparing and contrasting caspase-1 to apoptosis. For example, mediators of apoptosis, like the Bcl family members are caspase-1 inhibitors and may function to balance HSC activation and HSC cell death. An example of how we would test a hypothesis of the direct role of caspase-1 in HSC activation:

__________________________________________________________________

A. Specific Aims:

Nonalcoholic fatty liver disease (NAFLD) progresses through a series of pathologies including steatosis. Steatosis advances to nonalcoholic steatohepatitis (NASH), which is characterized by inflammation and hepatocellular cell death [167]. Caspase-1, a pro-inflammatory caspase has been recently implicated in NASH liver injury. Caspase-1 and its activating complex, the NLRP3
inflammasome are upregulated in mice following methionine and choline deficient (MCD) diet [102,138], high fat diet [102], or LPS administration [139]. We have demonstrated that mice deficient in caspase-1, Casp1−/− are protected from MCD-induced inflammation and fibrogenesis. Recent data of Casp1−/− mice on a long-term high fat diet suggest a protection from obesity, steatosis, inflammatory cytokines and early fibrogenesis markers (Dixon et al, under review).

The caspase-1 activating complex is present in both quiescent and activated hepatic stellate cells (HSC) and once activated, contributes to their ex vivo activation [136]. However, there are no data to suggest a mechanism of physiological HSC activation that is dependent on the inflammasome. In preliminary studies, we find, for the first time, increases in the inflammasome component expression in HSC following LPS stimulation ex vivo.

Based on the observation that caspase-1 is expressed during HSC transdifferentiation, we propose that HSC activation during liver injury is caspase-1-dependent (See Figure 1). This proposed work will examine the contribution of caspase-1 to HSC activation and function, a novel mechanism that would uncover a new molecular target for therapeutic intervention.
Hypothesis: Hepatic stellate cell caspase-1 plays a direct role in the regulation of transactivation and functions of differentiated HSC.

**Aim 1:** To determine if caspase-1 activation contributes to HSC transdifferentiation required for fibrogenesis *ex vivo*. We will address the contribution of caspase-1 in HSC transdifferentiation using a combination of methods to inhibit caspase-1: HSC from *Casp1*" mice, pharmacological inhibition and siRNA. We will also examine the role of caspase-1 in the molecular transdifferentiation programming involving the regulation of PPARγ.

**Aim 2:** To investigate the molecular mechanisms of caspase-1-dependent HSC functions. Using various modulators of HSC *ex vivo*, we will identify the underlying mechanisms by which caspase-1 contributes to the phenotypic responses of activated HSCs, such as proliferation, matrix degradation, chemoattraction and contractility.
Figure 1: Schematic presentation of the proposed hypothesis, describing molecular events of caspase-1-mediated HSC transdifferentiation and functions.
B. Research Strategy:

**B1. Significance:** The main cell responsible for hepatic fibrosis is the hepatic stellate cell (HSC). Upon liver injury, HSCs become activated and transdifferentiate into extracellular-matrix secreting myofibroblast-like cells. Mechanisms of HSC activation and transdifferentiation are complex and are incompletely understood. HSCs respond to immune cells, such as Kupffer cells and modulate the hepatic immune response [168]. The inflammasome is a multimeric protein complex that plays an important role in immune cells recognition of danger signals associated with cellular injury and death [80]. Its effector molecule is caspase-1, an enzyme responsible for the cleavage and activation of pro-inflammatory cytokines IL-1 and IL-18 [73].

Recently, the inflammasome has been identified in HSC that were activated ex vivo on plastic. Uric acid crystals activate the NLRP3 inflammasome in human HSC cell line, LX2 cells and primary wildtype murine HSCs [136]. Data suggest that the inflammasome contributes to HSC transdifferentiation LX2 cells and wildtype HSCs had increases in mRNA of TGFβ and collagen 1, as well as actin reorganization in response to uric acid crystals. Mice deficient in NLRP3 or ASC showed reduced responses to uric acid crystals. Uric acid crystals also inhibited HSC calcium signaling and migration in wildtype HSCs. We have found in preliminary experiments that mRNA for inflammasome components are upregulated upon activation of primary HSC from wildtype mice (see Figure 2).
Moreover, we have a pilot experiment in which the inflammasome components are further increased in response to LPS (see Figure 3).

In addition, there is evidence that the inflammasome is necessary for fibrogenesis and fibrosis. Inflammasome component (NLRP3 and ASC) deficient mice are protected from both thioacetamide and chronic, 8-week, high dose carbon tetrachloride -induced fibrosis [136]. In contrast, our experiments with caspase-1 deficient mice (Casp1−/−) did not confer protection from an acute 72-hour or even 5-week carbon tetrachloride protocol. Clearly the mechanisms of HSC transdifferentiation and function, including fibrogenesis are complex and more studies are needed to fully understand the implications of the inflammasome in HSCs because all of the functions of activated HSCs contribute to liver injury. 

**We hypothesize that hepatic stellate cell caspase-1 plays a direct role in the regulation of HSC transactivation and specific HSC functions.**

**B2. Innovation:** HSC activation is the major regulator of fibrogenesis in many types of liver injury, including alcoholic liver disease, non-alcoholic liver fatty liver disease and viral infection. Therapeutic strategies to limit or inhibit HSC transactivation and function exist, but are limited in their efficacy. In addition, there exists a second area of HSC research, which is focused upon the reversal of HSC to their quiescent phenotype and mechanisms of HSC cell death. Caspase-1 is activated upon liver injury in many hepatic cells, such as Kupffer cells, hepatocytes and HSCs. Therefore, studies of the inflammasome and caspase-1
activity in isolated cell types, such as HSC will help us understand molecular mechanisms of fibrogenesis and analysis of a novel therapeutic target.

C. Approach:

C1. Preliminary data: Inflammasome expression is upregulated upon activation of primary wildtype HSCs ex vivo: Primary HSCs activate in culture after 7 days [169]. We hypothesized that the activation of primary HSCs induces the expression of the inflammasome ex vivo. Analysis of the activation of primary wildtype HSC ex vivo demonstrates the increased expression of mRNA for αSMA and COL1α1 on days 2, 5 and 10 of culture (Figure 2A). Expression of mRNA for the inflammasome components, ASC, NLRP3, Casp1 and IL-1 were also examined at days 2, 5 and 10 of culture (Figure 2B). These preliminary data suggest that activation of primary wildtype HSCs ex vivo is associated with increases of NLRP3 and IL-1 mRNA expression (n=2). Analysis of ASC and Casp1 (n=2) expression also indicates a more modest increase at the mRNA level, but additional studies are needed to confirm inflammasome component expression during HSC transdifferentiation. Although there is a relationship between the inflammasome and HSC activation [136], there is a dearth of evidence to show an inflammasome-dependent molecular mechanism.
Figure 2. **Markers of HSC activation are increased after 5 to 7 days of culture and are associated with the increase of inflammasome components.**

Primary HSCs isolated from C57BL/6 wildtype mice (6-8 weeks old) were cultured for up to 10 days. After 2, 5 and 10 days, RNA was harvested, and real-time PCR was performed for analysis of the expression of mRNA for HSC activation markers αSMA and COL1α1. n=5 (A) and inflammasome markers ASC, NLRP3, Casp1 and IL-1. n=2 (B). mRNA was quantified using the delta delta Ct method over 18S. Values are represented as fold change over day 2. Letter superscripts designate significance between groups. *P*<0.05. Number superscripts designate fold change from Day 2. Even though n=2, the data are displayed as mean±SEM for convenience.
LPS stimulation of primary HSCs is associated with inflammasome activation. Lipopolysaccharide (LPS), a component of the outer wall of gram-negative bacteria signals through TLR4, has been implicated in the pathogenesis of NAFLD [45]. Excess dietary intake of fructose and FFA contributes to altered gut flora motility, growth, and increased intestinal permeability, resulting in portal circulation of LPS [46]. Ganz et al. recently reported that LPS, a ligand for TLR4, may be an important regulator of hepatic expression of caspase-1 and the NLRP3 inflammasome [139]. In addition, HSCs express TLR4 [155]. We hypothesized that LPS could induce the expression of the inflammasome in HSC ex vivo. HSC from wildtype mice have increased mRNA expression for inflammasome protein NLRP3, Casp1 and IL-1 after an overnight stimulation with LPS (Figure 3A). It has been shown that LPS stimulation of HSCs upregulates mRNA for IL-6 and IL-8 [170,171], which we confirmed in primary mouse HSCs ex vivo (Figure 3B).
Figure 3. **Inflammasome components are upregulated at the mRNA level after LPS stimulation of wildtype primary HSCs ex vivo.** NLRP3, Casp1 and IL-1 mRNA is increased after overnight LPS treatment. n=2 (A). Primary HSCs respond to LPS treatment and increase mRNA expression of IL-6 and IL-8. n=2 (B). mRNA was quantified using the delta delta Ct method over 18S. Values are represented as fold change over CTL. Even though n=2, the data are displayed as mean±SEM for convenience.
These pilot data suggest that the inflammasome is activated and contributes to HSC transactivation \textit{ex vivo}. Mid-activation phase HSC also upregulate inflammasome components after LPS stimulation, suggesting an important physiological response of the inflammasome during transdifferentiation. This proposal will investigate the role of caspase-1 and the inflammasome in HSC transactivation. Specifically, we will assess the mechanism of caspase-1/PPARγ-mediated HSC activation. We will also propose to explore molecular mechanisms for the contribution of caspase-1 to specific functions of HSCs.

**Experimental Strategy**

1. **Aim 1: To determine if caspase-1 activation within HSC, contributes to HSC transdifferentiation required for fibrogenesis.** Our preliminary results suggest that the inflammasome components are expressed during primary wildtype HSC activation \textit{ex vivo} and are sensitive to LPS stimulation (Figure 2 and Figure 3). These results suggest a role for caspase-1 in the activation and transdifferentiation of HSCs. We therefore hypothesize that **caspase-1 is directly activating HSCs**, contributing to the transdifferentiated HSC phenotype. If caspase-1 is involved in direct regulation of HSC transdifferentiation and functions required for fibrogenesis, then inhibition or deficiency of caspase-1 would prevent HSC activation.

**Experimental strategy: 1A. To determine if deficiency or inhibition of caspase-1 prevents HSC transdifferentiation:** First, we will determine the
temporal peak of caspase-1 expression and activation during HSC transactivation. To do this, we will treat primary wildtype HSCs with LPS at days 2, 5, and 10 of HSC culture. DMEM media will be used as a control. We will then use quantitative real-time PCR (qRT-PCR) to determine the mRNA expression of caspase-1. We will also measure caspase-1 activity. Another way in which we will assay caspase-1 activity in treated HSCs is by western blot to determine expression of IL-1 and IL-1β. We will then determine the transactivation state of the HSCs ex vivo. Markers of HSC activation include mRNA for αSMA, COL1α1, and TGFβ will be measured by qRT-PCR. We will also use αSMA immunocytochemistry to determine HSC morphology. Next, to study the effects of caspase-1 on HSC transdifferentiation, we will use primary HSCs isolated from caspase-1 deficient mice (*Casp1−/−*) [107]. If caspase-1 is involved in HSC transactivation, the deficiency of caspase-1 will prevent this process. We will also use a tetrapeptide aldehyde acyl-Tyr-Val-Ala-Asp-CHO, YVAD-CHO (Cayman Chemicals), which forms a thiohemiacetal with the active cysteine site of caspase-1 and prevents release of IL-1β. Cells treated with DMSO will act as a control. A robust strategy to knockdown caspase-1 in primary HSCs would to transfec HSCs with siRNA using Oligofectamine Reagent (Invitrogen). Cells treated with a scrambled siRNA will act as a control. The inhibitor and siRNA strategies will allow us to determine precisely when during the transdifferentiation caspase-1 is involved. To confirm the inhibitor or siRNA is suppressing caspase-1 activity, we can employ the caspase-1 activity assay or IL-1 and IL-1β western blot. Then we
will continue to investigate HSC transactivation phenotypes in the \textit{Casp1}^{-/-} HSCs or the cells treated with pralnacasan or siRNA.

**1B. To determine if caspase-1 is contributing to an anti-adipogenic phenotype of HSC, promoting transdifferentiation:** Mechanistic investigations of HSCs show that the retinoid-containing quiescent cells have an adipogenic phenotype. PPAR\(\gamma\) and a number of adipogenic transcription factors, including C/EBP, LXR and SREBP-1, maintain HSC quiescence. This adipogenic programming is lost during HSC transactivation [172]. Caspase-1 is known to degrade PPAR\(\gamma\) protein in adipocytes [173]. Therefore our hypothesis is that caspase-1 inhibits HSC adipogenic transcriptional regulation, resulting in HSC activation and a pro-fibrogenic fibroblast. Outcomes from Aim1A will direct our research experiments in 1B. Based on the timing of caspase-1 activation in Aim1A, we will determine if activation of caspase-1 contributes to PPAR\(\gamma\) degradation and the downstream signaling events of PPAR\(\gamma\) inhibition, such as activation of TGF\(\beta\) and PDGF signaling pathways for pro-fibrogenesis by western blot. These events will be investigated for caspase-1 dependence through the use of \textit{Casp1}^{-/-} HSCs, YVAD-CHO, and Casp1 siRNA and corresponding controls.

**Expected outcomes/alternative strategies: 1A.** Based on our preliminary results, we expect that caspase-1 will be upregulated during the mid-activation phase of HSCs. Treatment of HSCs with LPS will upregulate mRNA of caspase-1, caspase-1 protein, increase caspase-1 activity and the processing of IL-1 to IL-1\(\beta\).
Mid-activation phase HSCs will upregulate mRNA of αSMA, COL1α1, and TGFβ. Treatment of HSCs will upregulate them furthermore, indicating a connection between caspase-1 and transactivation markers of HSCs.

The Casp1−/− HSCs or the cells treated with YVAD-CHO will have no processing of IL-1 to IL-1β. siRNA against caspase-1 turned on during the mid-activation phase would have the same result as the knockout cells or the YVAD-CHO treated cells. However, it is more likely that with siRNA against caspase-1 during the quiescent phase would have a more dramatic effect of inhibiting the transactivation of HSCs, whereas siRNA against caspase-1 during the late-activation phase would have less effect. These results would indicate that caspase-1 is necessary for the mid-phase of HSC transdifferentiation.

If YVAD-CHO does not effectively inhibit caspase-1 in HSCs, a variety of commercially available inhibitors could be used as alternatives, including a potent and selective irreversible caspase-1 inhibitor, α-substituted ketone chloromethylketone (YVAD-CMK) (Cayman Chemicals). A possible pitfall of the siRNA strategy to inhibit caspase-1 is that transfecting primary HSCs is difficult. We will use the human hepatic stellate cell line LX2 as an alternative cell type transfected with Oligofectamine Reagent (Invitrogen). LX2 cells are immortalised human stellate cells that have high transfectability. However, the use of primary HSCs is preferred for most HSC studies because the LX2 cell line is 1) of human origin instead of mouse, 2) immortalised and 3) do not follow the
same transactivation pathways as primary cells. LX2 cells are activated when they attach to plastic. Therefore, it may be difficult to study the role of caspase-1 in transactivation using LX2 cells.

**1B.** Recently work has been done on caspase-1-mediated adipocyte differentiation. Steinstra et al. shows that caspase-1 is upregulated during adipocyte differentiation [174]. Our hypothesis is that caspase-1 contributes to an anti-adipogenic transcriptional regulation of HSCs, and cause HSC transdifferentiation. It is possible that the upregulation of caspase-1 in HSCs could maintain a quiescent phenotype of HSCs, similar to adipocyte differentiation, mediated by PPARγ. Therefore, we would reverse our proposed experiments to test HSC transdifferentiation to experiments focused on HSC reversion. HSC reversion is a topic of interest because this is a means to reversing fibrogenesis and maintaining a quiescent HSC phenotype. These experiments would involve activating HSCs *ex vivo* and inducing caspase-1 expression. The readout would be PPARγ activation and other adipogenic transcription factors, such as C/EBP and SREBP-1 by western blot.

**2. Aim 2: To investigate the molecular mechanisms of caspase-1-dependent HSC functions.** Using various modulators of HSC *ex vivo*, we will identify the underlying mechanisms by which caspase-1 contributes to the phenotypic responses of activated HSCs, such as proliferation, matrix degradation, chemoattraction and contractility.
Experimental strategy: 2. To determine the effects of caspase-1 on HSC proliferation, matrix degradation, chemoattraction and contractility functions. A previous study explores the effect of the inflammasome on HSC chemotaxis [136], but further work is needed to fully understand the impact of caspase-1 on all HSC functions required for fibrogenesis.
<table>
<thead>
<tr>
<th>Stimulus</th>
<th>HSC Function</th>
<th>Functional Indicator</th>
<th>Possible Upstream Mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF</td>
<td>Proliferation</td>
<td>BrDU, ki67</td>
<td>MAPK, JNK, ERK</td>
</tr>
<tr>
<td></td>
<td>Chemotaxis</td>
<td>migration/scrape assay</td>
<td>PI3K, Akt</td>
</tr>
<tr>
<td></td>
<td>Chemoattraction</td>
<td>Boyden chamber assay</td>
<td>MCP-1</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Fibrogenesis</td>
<td>qRT-PCR for αSMA, COL1α1</td>
<td>SMAD, MAPK, COL1</td>
</tr>
<tr>
<td></td>
<td>Retinoid Loss</td>
<td>Morphology on day 2</td>
<td></td>
</tr>
<tr>
<td>ET-1</td>
<td>Contractility</td>
<td>Collagen matrix shrinkage</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Endpoints for HSC function experiments
To understand the effects of caspase-1 on specific HSC functions, including proliferation, chemotaxis, contractility, fibrogenesis, matrix degradation, retinoid loss and chemoattraction, caspase-1 will be suppressed. The best outcome from the different methods of inhibiting caspase-1 in Aim 1A will be employed here. In addition to Casp1−/− HSC, pralnacasan or siRNA, we will add stimuli of specific HSC functions and determine their respective readouts. PDGF will be added to HSCs to induce proliferation. Primary readouts of proliferation include MAPK, JNK and ERK activation by western blot. In addition, immunocytochemistry will be performed for ki67, a proliferation marker. PDGF also induces HSC chemotaxis. Therefore the readouts for chemotaxis include PI3K and AKT by western blot. To study contractility, we will culture HSCs on a collagen-coated matrix and treat HSCs with ET-1. The distance that the matrix contracts is a direct readout of HSC contractility. To determine the effects on fibrogenesis, we will treat HSCs with TGFβ. The readout for fibrogenesis is the phosphorylation of SMAD and MAPK by western blot. In addition, qRT-PCR will be performed to determine collagen synthesis by measuring mRNA for COL1α1. We can also look at matrix degradation and retinoid loss via TGFβ. Matrix degradation readouts would be qRT-PCR analysis of mRNA expression for MMP-2 and TIMP-1. Retinoid loss will be examined at day 2 of culture in every experiment via microscopy. Chemoattraction will also be assessed via PDGF. qRT-PCR will be performed for chemokine mRNA expression of MCP-1.
**Expected outcomes/alternative strategies:** 2. Assessing specific HSC functions will help us understand which HSC processes caspase-1 is affecting. We expect that knockdown or inhibition of caspase-1 will prevent the effects of PDGF, ET-1 and TGFβ on HSC proliferation, chemotaxis, contractility and chemoattraction. If caspase-1 does not have an effect on HSC transdifferentiation or functions of activated HSCs, we will explore the hypothesis that IL-1 and/or IL-18 mediate these processes. The administration of IL-1Ra, or anti-IL1 or anti-IL18 antibody will help answer these questions. Based on results from Aim 1, and if neither caspase-1, IL-1 or IL-18 have an effect on activated HSC function, we will conclude that these inflammasome mediators are causative of HSC transactivation in the initiation phase of fibrogenesis instead of perpetuation and HSC function.

In addition, another means of caspase-1 to reverse fibrosis is caspase-1–mediated pyroptosis and HSC cell death. Pyroptosis is a programmed cell death similar to apoptosis, but is morphologically like necrosis. The characteristics of pyroptosis are cell lysis, cell swelling, pore formation, DNA fragmentation and caspase-1 activation. These events are independent of caspase-3 and cytochrome c release. For these experiments, activated HSCs on day 7 or 10 in culture will be treated with LPS to induce caspase-1 activation. The HSCs will then be assessed for the release of intracellular contents such as lactate dehydrogenase and inflammatory mediators like TNFα, IL-6 and IL-8 into the conditioned medium by ELISA. In addition, TUNEL staining will be performed to determine cell death.
**Data analysis:** Unless noted otherwise, statistical analysis will be performed using SAS (Cary, NC). Mean± SEM values will be calculated for each experimental group. Unpaired Student’s t-test will be used to compare two groups. Comparisons between multiple groups will be analyzed using general linear models (Two-way ANOVA) with a Least squared means to test significance between individual groups and identify interaction. Probability levels will be considered significant when $p < 0.05$. Four independent experiments with triplicates are required for all statistical analysis.
III. Clinical Correlate

It is estimated that there are 20% of people in the United States have NAFLD, of which 3-5% have NASH. The spectrum of NAFLD includes steatosis, steatohepatitis with or without fibrosis, cirrhosis and hepatocellular carcinoma. Steatosis and early steatohepatitis are reversible. However, there are no approved treatments for NAFLD. Therefore, it has become imperative to prevent NAFLD progression. Clinical recommendations are to reduce BMI, increase activity and limit alcohol and medication usage [175].

IL-1β has been implicated in a number of pro-inflammatory processes, including systemic juvenile rheumatoid arthritis [68]. It is standard for therapeutics to target the most distal point of the disease, in this case IL-1β. Treatment with IL-1 Receptor antagonist (IL-1Ra) significantly improves symptoms of rheumatoid arthritis and is without complications in humans [68,176]. Emerging data provide evidence that IL-1β is implicated in NAFLD pathogenesis [4,5]. Anakinra, a recombinant human IL-1Ra is used clinically to treat patients with NAFLD. However, no significant studies have been done to show the efficacy and safety of anakinra in NAFLD. Other agents, such as IL-1 Trap, IL-1β monoclonal antibodies and IL-1R1 monoclonal antibodies may prevent IL-1β-mediated disease [177]. These encompass several levels of control of the synthesis, processing and secretion of IL-1β. In addition, once IL-1β is released, it must compete for the binding of its receptor with endogenous IL-1Ra, IL-1 type II decoy receptor and IL-1R accessory protein [178]. All of these
mechanisms inhibit IL-1β and may also be useful in therapeutic strategies of NAFLD.

Another strategy to limit inflammation and action of IL-1β is to use caspase-1 inhibitors. This strategy has advantages over inhibiting the IL-1β cytokine [177]. First, protein-based products against IL-1β cytokine, while effective, are administered via injection. Injections are inconvenient for patients and they also cause adverse side effects. The development of a low-molecular weight protein, orally administered anti-cytokine therapy is highly sought after. A new selective caspase-1 inhibitor, Pralnacasan reduces experimental murine rheumatoid arthritis [179] and murine colitis [180]. However, there are no data to suggest the efficacy and safety of caspase-1 inhibitors in human disease. The Investigation of the Vertex drug VX-740 has stopped in the clinic, while pan-caspase inhibitor VX-765 has advanced to Phase I [181]. In theory, a caspase-1 inhibitor will be just as effective at reducing inflammatory diseases as IL-1Ra. However, it may have an added bonus by inhibiting the processing of IL-18 in addition to IL-1β. Speculative negative consequences of caspase-1 inhibitors would be a reduction in immune responses, resulting in increased susceptibility to infection and illness. However, the short half-life of caspase-1 inhibitors would be cleared rapidly if side effects occur. Therefore, it is interesting to follow the development of caspase-1 inhibitors and to see if they are proposed or used in the treatment of NAFLD.
IV. Conclusion

This thesis has addressed the role of caspase-1 in metabolic dysregulation liver and adipose in NASH pathogenesis. Caspase-1 plays important roles in many cell types of the liver, especially hepatocytes, KCs and HSCs. Diet models of NASH have explored the effect of caspase-1 on different stages of NASH injury, including steatosis, inflammation, and fibrogenesis. Additional studies will further elucidate cell-specific mechanisms of caspase-1 activation and its effects on NASH liver injury. There are many potential therapeutic implications of this knowledge to impact the health and lives of patients with NASH.


Acceleration of Early Markers of CCl(4) -Induced Fibrosis but not Steatosis or Inflammation. Alcohol Clin Exp Res: 36(7):1139-47.


