UNIVERSITY OF CALIFORNIA SAN DIEGO

Activation of Liver X Receptors in Kupffer Cells Modulates Inflammatory Transcriptional Programs During Liver Disease

A Thesis submitted in partial satisfaction of the requirements
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in

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by

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The Thesis of Cassi Molly Bruni is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

This Master's thesis is dedicated to the Glass lab members for all their support and the Glass lab for creating an exciting and productive laboratory environment.

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LIST OF ABBREVIATIONS

ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
DAMPS	Damage-Associated-Molecular-Pattern Molecules
DKO	Double Knockout
ECM	Extracellular Matrix
FACS	Fluorescence Activated Cell Sorting
FDR	False Discovery Rate
HDL	High Density Lipoprotein
HSC	Hepatic Stellate Cell
GO Term	Gene Ontology Term
KC	Kupffer cell
KO	Knockout
FC	Fold Change
LPS	Lipopolysaccharide
LXR	Liver X Receptor
LXRα	Liver X Receptor Alpha
LXRβ	Liver X Receptor Beta
Lyz2	Lysozyme 2
MMPs	Matrix Metalloproteinases
NAFL	Nonalcoholic Fatty Liver
NALFD	Nonalcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis

NF-KB	Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells
PAMP	Pathogen-Associated-Molecular-Pattern
PCR	Polymerase Chain Reaction
PPARα	Peroxisome Proliferator-Activated Receptor Alpha
qPCR	Quantitative Polymerase Chain Reaction
RCT	Reverse Cholesterol Transport
RNA-seq	RNA-Sequencing
RXR	Retinoid X Receptor
TF	Transcription Factor
TLR4	Toll-Like-Receptor 4
TPM	Transcripts Per Million

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ABSTRACT OF THE THESIS

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by

Cassi Molly Bruni

Master of Science in Biology

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Professor Christopher K. Glass, Chair
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Liver X Receptors (LXRs), composed of LXRα and LXRβ, are ligand activated nuclear receptors that are important regulators of cholesterol and lipid metabolism. LXRs control expression of genes involved in cholesterol efflux, fatty acid metabolism, and inflammation in tissues such as the liver and intestine. LXRα is highly expressed in liver resident macrophages, called Kupffer cells, which are involved in clearing pathogens from the portal circulation, as well as sensing tissue injury. During liver disease, such as nonalcoholic steatohepatitis (NASH),

Kupffer cells are responsible for initiating a proinflammatory response program; therefore, I sought to understand the LXR-dependent responses modulating cholesterol accumulation and inflammation in Kupffer cells at both the steady state and during disease. I found a decreased expression of cholesterol efflux and inflammatory genes in Kupffer cells of mice lacking LXRs throughout all tissues but aimed to understand the function of LXRs intrinsic to Kupffer cells. Therefore, I studied a Lyz2 Cre and a novel Clec4f Kupffer cell specific Cre mouse lacking LXRs and saw an expansion of a new F4/80^{Hi}Cd11b^{Int}Tim4^{Neg} Kupffer cell population that is associated with fatty liver disease. Consequently, LXR activation may have beneficial properties, so pharmacological activation with a synthetic mimetic of the natural LXR ligand desmosterol was analyzed. Results suggest that this desmosterol mimetic dampens hepatic inflammation, lipid accumulation, and fibrosis, and may not only be targeting the liver, but also altering gene expression in the intestine as well. Thus, pharmacological activation of LXRs may act therapeutically in patients with fatty liver disease.

CHAPTER 1. INTRODUCTION

1.1 Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis

With the increasing prevalence of sedentary lifestyles and high-calorie diets, nonalcoholic fatty liver disease (NAFLD) is becoming more common, estimated to be affecting around one billion people [1,2]. NAFLD is associated with obesity, diabetes mellitus, and dyslipidemia, which overlap with the characteristics of metabolic disease. A high percentage of people who are living with diabetes mellitus or severe obesity also have fatty liver disease [3,4]. NAFLD encompasses a spectrum of liver diseases and is mainly defined by hepatic fat accumulation, or steatosis, in patients without substantial alcohol consumption. In the United States, NAFLD is thought to affect roughly one-third of adults and is the most common cause of chronic liver disease [2,5]. In 2016, the annual burden of NAFLD in the United States was $103 billion and is predicted to increase drastically in the near future [6].

NAFLD can be divided into two subgroups: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFL consists of simple steatosis and is considered to be benign; however, NASH is more severe and is defined by lobular inflammation, hepatocellular damage and fibrosis, in addition to steatosis [1]. As a distinguishing feature of this disease, steatosis, is the buildup of excess lipid resulting in fat deposition within hepatocytes, causing the enlargement of the cell and formation of lipid droplets [7]. NASH is more likely to occur in patients who have a form of NAFLD, as 25 percent of patients at the NAFLD stage eventually progress to NASH, which puts them at a high risk for developing cirrhosis, liver cancer, or even liver failure [5]. NASH is currently the second most common reason for liver transplant behind hepatitis C, but is predicted to become the leading cause by 2020 [8].

Liver fibrosis, lobular inflammation, hepatocellular ballooning, and steatosis are the characteristics for defining NASH, and are usually identified by histological analysis of tissue collected from liver biopsies. A liver biopsy is an invasive procedure that is currently the best method for NAFLD and NASH diagnosis, as there are no adequate biomarkers for the identification of this disease. There are available serum tests; however, they are not sufficient for detecting the middle stages of NASH. New techniques, such as magnetic resonance elastography, an imaging method that measures the stiffness of tissue, are being optimized to detect NASH [9]. Early detection allows for the best management of NAFLD because the liver can regenerate and heal if preventative measures are taken. Due to this being an invasive procedure, there is less widespread use of this method and it is usually selective for patients with a higher chance of being diagnosed with a more severe form of NASH [5]. Therefore, there is no simple test to check if a patient has a mild form of NAFLD. This reveals the need to develop unique noninvasive biomarkers for early diagnosis of NASH [5]. Although it affects many people, there is also currently no approved therapeutic agent for the treatment of this disease, leaving room for the investigation and advancement of this field.

1.2 Cellular mechanisms governing the progression of nonalcoholic steatohepatitis

Chronic inflammatory conditions that occur during NASH disrupt the homeostatic liver environment and causes an alteration in the myeloid cell populations that contribute to the disease phenotype. The liver is an extremely heterogeneous organ with diverse cell populations that respond to disease signals to produce the NASH phenotype, which includes the molecular mechanisms that induce fibrosis, steatosis, lobular inflammation, and hepatocellular ballooning. The liver is the first filter system for the blood traveling from the intestine through the portal vein that contains microbial products and lipopolysaccharide (LPS) from bacteria in the gut. The

liver contains the largest population of macrophages in the body that play vital roles in responding to these products and signaling during NAFLD and NASH [10].

Macrophages are innate immune cells that function to maintain tissue homeostasis by removing dead cells and killing pathogens [10]. They reside in all tissues throughout the body and have specialized functions, such as detoxification of microbial products from the intestine by Kupffer cells, maintenance of bone homeostasis by osteoclasts, and neuronal synaptic remodeling by microglia [11]. Macrophages respond to a wide range of external stress signals, making them essential for the disease response. The origin of macrophages can partly explain the cellular heterogeneity that occurs during NASH. The field is currently debating the origin and differentiation of macrophages; however, it is known that macrophages can arise from multiple sources and may have different roles based upon where they originate [12]. This includes circulating monocytes, that are recruited during liver injury, and tissue resident yolk sac precursor derived macrophages called Kupffer cells [10].

Kupffer cells, located in the lumen of the liver sinusoidal endothelium, are essential for sensing tissue injury and initiating inflammatory responses in the liver. At the steady state they function to clear senescent red blood cells, sense drainage from the portal system, detoxify LPS, and respond to a diverse array of biological signals. During NASH, Kupffer cells respond to severe liver injury by generating a wound healing proinflammatory response program that results in the accumulation of scar tissue. They secrete inflammatory signals, which increases the diversity of hepatic macrophages by recruiting monocyte derived macrophages that influence disease progression and resolution [13]. Kupffer cells become activated by Damage-Associated-Molecular-Pattern molecules (DAMPS) that are released by damaged hepatocytes. It is currently thought that NASH begins with the buildup of fatty acids and cholesterol in hepatocytes, which

causes damage to these cells. The Kupffer cells then release cytokines such as *IL-1b* and *TNF* that increase damage by inducing apoptosis [10]. LPS, which is a Pathogen-Associated-Molecular-Pattern molecule (PAMP) also stimulates Kupffer cells in addition to hepatic stellate cells (HSC) through Toll-like-receptor 4 (*TLR4*). When activated, stellate cells produce collagen which leads to deposits of scar tissue throughout the liver, called fibrosis. Increased levels of *Ccl2* promotes the infiltration of monocytes that enter the liver and develop a pro-inflammatory phenotype which causes areas of densely packed cells, including monocytes and neutrophils, known as lobular inflammation. Simultaneously, there is a build-up of triglycerides in hepatocytes, called steatosis, due to the induction of de novo lipogenesis and the suppression of peroxisome proliferator-activated receptor alpha (*PPARα*) in macrophages [10,13]. The last feature present during NASH is called hepatocellular ballooning, which occurs when the hepatocytes become enlarged and swollen, as their cytoplasm expands [14]. This occurs due to the accumulation of fat droplets in the cytoplasm that causes a rearrangement of the cellular compartments. Macrophages play a central role in contributing to the cellular mechanisms governing the progression of NASH, so studying macrophages during NASH may give some insight into this complicated disease.

Adding to the complexity of the liver environment, Kupffer cells can also play an antifibrotic role during the progression of NASH. Hepatic fibrosis is an interesting feature of NASH because it represents a wound-healing response mechanism to acute or chronic liver injury that causes the accumulation of extracellular matrix (ECM). It is also a clinically relevant predictor of liver related mortality, as patients with fibrosis are more likely to die from their liver disease. If the injury is acute, then the liver is able to regenerate and restore the healthy tissue and architecture, which is possible at the less severe stages of NAFLD. However, repeated and

prolonged injury leads to a large buildup of ECM and causes irreversible scar tissue to form [15]. Kupffer cells are integral to these processes, as they can signal the production of matrix metalloproteinases (MMPs) with the release of chemokines to destroy collagen and resolve the scar tissue. MMPs are a family of calcium-dependent enzymes that degrade collagenous and noncollagenous ECM which can begin to reverse liver fibrosis [15]. Thus, Kupffer cells can adopt different programs to either promote fibrogenesis or fibrosis resolution. Many questions on how Kupffer cells and other hepatic macrophages are involved in NASH still remain due to the heterogeneity of the liver.

1.3 Liver X receptors as master regulators of lipid and cholesterol homeostasis

As discussed in the previous section, Kupffer cells are integral to the disease progression of NASH. Therefore, understanding the factors that govern this cell type and its identity may help elucidate how Kupffer cells contribute to and play a role in the development and mitigation of NASH. Tissue resident macrophages have differing sets of gene expression programs that establish their identities. It has been shown that Liver X receptors (LXRs) are involved in establishing Kupffer cell identity due to distinct enhancer landscapes [16]. In addition to being thought of as a lineage determining transcription factor (TF) for Kupffer cells, LXRs are further related to NASH due to their role as master regulators of cholesterol export. During NASH, the capacity of the liver for processing metabolites becomes exceeded, which leads to the accumulation of toxic lipid metabolic substrates. This exacerbates the hepatocyte injury and hepatic inflammation occurring during the variety of molecular pathways that are progressing the disease. In human NASH, free cholesterol levels are elevated [17], which also occurs in mice, as feeding them a high fat and cholesterol diet will induce hepatic inflammation [7]. There has been a growing interest in the field for the importance of cholesterol metabolism and cholesterol

burden as a contributor to hepatic homeostasis and metabolic disease [18]. LXRs appear to play a key role during disease, so investigating the functions of LXRs, especially in Kupffer cells, may reveal more about the underlying mechanisms contributing to the complex diseases of NAFLD and NASH.

Liver X Receptors (LXRs), composed of the forms LXRα (*Nr1h3*) and LXRβ (*Nr1h2*), are ligand activated nuclear receptors that are involved in the transcriptional control of lipid metabolism. They are members of the nuclear receptor superfamily, which are responsible for transferring hormonal, metabolic, and nutritional signals into changes in gene expression. They consist of a DNA-binding domain (DBD) and a ligand-binding domain (LBD), which when bound to the specific ligand, swap co-repressors for co-activators in order to translate a signal [19]. LXRs bind to their response element LXRE located in their promoter regions as heterodimers with the Retinoid X Receptor (RXR). LXRα is expressed in metabolic tissues including the liver, intestine, kidney and adipose tissue, while LXRβ is found throughout the body [20]. Interestingly, LXRα is highly expressed in Kupffer cells, which as mentioned above, are involved in clearing pathogens from the portal circulation, as well as initiating inflammatory responses. LXRs are master regulators of cholesterol and lipid homeostasis and control the expression of genes that encode proteins involved in cholesterol efflux, transport, excretion, and conversion to bile acids [21].

A major way LXRs are involved in the movement of cholesterol is by a process called reverse cholesterol transport (RCT), in which excess cholesterol from peripheral tissues is returned to the liver by high density lipoprotein (HDL). LXRs stimulate this process by activating the transmembrane proteins *Abca1* and *Abcg1*. The first paper to reveal that LXRα has a role in cholesterol metabolism showed that LXRα knockout (KO) mice lose their ability to

respond normally to dietary cholesterol and are unable to tolerate any amount of cholesterol in excess, along with impaired hepatic function [22]. Proper control of cellular and systemic lipid levels is critical for physiological homeostasis, as excessive cholesterol levels are toxic [18]. LXRs are also involved in inflammatory response pathways, as demonstrated by their ability to inhibit signal-dependent activation of proinflammatory transcription factors such as NF-kB, which decreases downstream pro-inflammatory gene expression [23]. LXRs have also been shown to suppress inflammatory response genes in macrophage foam cells present in atherosclerotic lesions [24]. Although the exact functions of LXRs are still unknown, they may have an anti-inflammatory effect on cells during the progression of NASH.

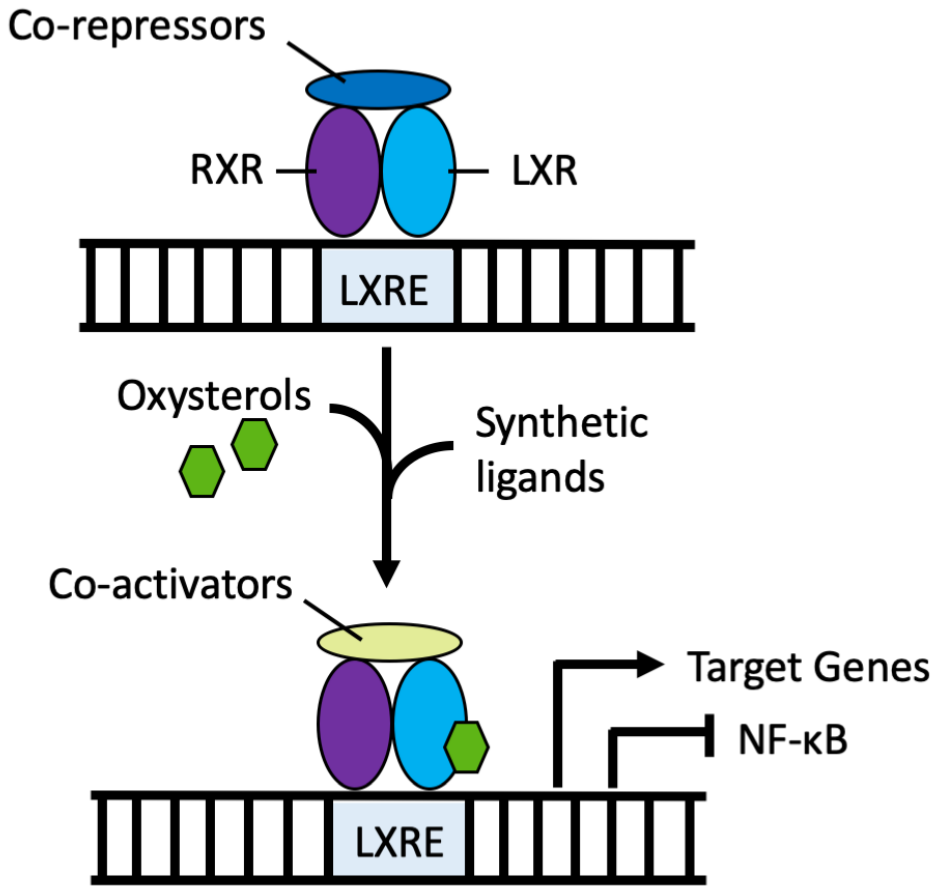

Figure 1. LXRs regulate genes involved in cholesterol efflux and homeostasis. Schematic showing the binding and activation of LXRs. LXRs are nuclear receptors that form a complex with RXR and co-activators or co-repressors. Ligands that bind to LXRs include oxysterols and cholesterol precursors. Downstream target genes are involved in cholesterol efflux, reverse cholesterol transport, and repression of inflammatory genes.

To better understand the role of LXRs, various studies have been performed using LXRα/β deficient mice. In one study, LXRα/β deficient mice were more susceptible to developing liver fibrosis when exposed to carbon tetrachloride or a methionine/choline-deficient diet. The activity of LXRs was linked to stellate cell function because stellate cells from LXRα/β DKO mouse livers displayed altered lipid droplet morphology and expressed higher levels of fibrogenic genes [25]. There is currently little knowledge about mice with LXRs absent in a

NASH context. Therefore, a NASH inducing diet given to mice lacking LXRs in a specific cell type may help determine the functions of LXRs during this disease. On the other hand, LXRs are activated by the endogenous ligands including oxysterols and certain intermediates in the cholesterol biosynthetic pathway, such as desmosterol. Most endogenous sterols that can activate LXRs also have the ability to inhibit the activation of the lipogenic transcription factor sterol regulatory element- binding protein 1c (*Srebp1c*) pathway, revealing that the LXR and SREBP pathways are coupled in controlling cholesterol homeostasis [18]. LXRs regulate fatty acid metabolism by increasing transcription of *Srebp1c* which turns on fatty acid synthase genes including fatty acid synthase (*Fasn*) and stearoyl-CoA desaturase 1 (*Scd1*) which contribute to hepatic lipogenesis [21]. This is a particularly important point for the following section discussing synthetic ligands that can bind to LXRs and activate them. In this thesis, I investigate the LXR-dependent responses modulating cholesterol accumulation and inflammation in Kupffer cells and how LXRs contribute to Kupffer cell function during NASH.

1.4 Potential therapeutic activity by activation of LXRs

As discussed in the previous section, LXRs are ligand-dependent transcription factors that form heterodimers with RXR and this complex can be activated by ligands of either partner [26]. Here, we will focus on LXRs and the ligands that bind and activate at the LXR binding site. LXRs bind oxysterols and intermediates of cholesterol biosynthesis, including molecules such as 27-hydroxycholesterol and desmosterol. They have long been thought of as attractive drug candidates because of their ability to promote cholesterol efflux and inhibit inflammation. Many different molecules have been synthesized over the years with the objective being to bind and activate LXRs. However, nearly all potent and selective synthetic LXR agonists have the unwanted side effect of inducing the expression of *SREBP1c*, which drives de novo lipogenesis

in the liver [27,28]. This results in hepatosteatosis and hypertriglyceridemia and is the main reason that existing synthetic LXR agonists have not been successfully advanced for clinical use. Recently, our laboratory identified desmosterol as the primary LXR ligand present in macrophage foam cells using metabolomic studies [24]. Desmosterol suppresses *SREBP1c* and the fatty acid biosynthesis pathway; therefore, it would be predicted that synthetic molecules resembling this natural endogenous ligand would selectively activate LXRs.

In 2004, Quinet et al performed a study using multiple synthetic mimetics, and identified one in particular, N, N-dimethyl-3β- hydroxycholenamide (DMHCA), that was able to activate LXR target genes in the liver, intestine and macrophages with limited effects on *Srebp1c* expression and serum levels of triglyceride [29]. This molecule resembles the structure of desmosterol more closely than other synthetic ligands that have been synthesized due to its similarity between side groups. Recent studies performed in our laboratory suggest that DMHCA selectively regulates LXR and *Srebp1c* in macrophages and Kupffer cells, but not in hepatocytes, as revealed through experiments of intraperitoneal injections of DMHCA [30]. These findings illustrate differences in the response of LXRs in various cell types, as well as the exciting potential of DMHCA as a new basis for future drug development. This synthetic mimetic has not been studied in depth in the context of NASH and may identify a new class of therapeutics for the treatment and prevention of this disease. Further, the studies of DMHCA in mice on the NASH model will provide insight into the role of LXR signaling and cholesterol homeostasis across hepatic, intestinal, and myeloid tissues during NASH pathogenesis.

LXRs not only regulate cholesterol in the liver, but also play a role in maintaining whole body cholesterol homeostasis in the intestine, as it is the path of entrance for cholesterol absorption into the body. Enterocytes are intestinal epithelial cells that highly express LXRs. In

the intestine, LXRs limit the uptake of cholesterol through the induction of genes such as *Abcg8/5*, which play a role in cholesterol excretion and the downregulation of genes such as *Npc1l1*, which regulates the absorption of cholesterol into the enterocyte [18]. A mouse model of increased LXR activity showed increased expression of *Abcg5* and *Abcg8* and increased fecal sterol excretion. Further, breeding these mice to Ldlr KO mice protected them against atherosclerosis when fed a high fat diet [31]. This raises the possibility that LXRs in the intestine may be a new promising drug target. Some intestine specific agonists of LXRs have been developed, such as GW6340, which promoted reverse cholesterol transport from macrophages in mice and significantly increased the excretion of sterol in the feces of these mice [32].

The activation of LXRs in the intestine appear to have beneficial effects on total body cholesterol as it leads to reduced intestinal cholesterol absorption, stimulation of RCT, and protection from atherosclerosis in the absence of hepatic steatosis [31]. This suggests that the intestine specific activation of LXRs may have beneficial effects on cholesterol levels and lend some protection from the development of NASH. There has recently been a growing interest in the importance of cholesterol metabolism in tissues other than the liver, making the role of the intestine as a contributor to cholesterol homeostasis and metabolic diseases an exciting new area of research. DMHCA was shown to be able to activate LXR target genes in the intestine, including *Abca1*, revealed by analyzing duodenal gene induction [29]. Therefore, studying the different regulatory pathways and molecular mechanisms affected by DMHCA across multiple tissues and cell types may be valuable when evaluating the potential utility of desmosterol mimetics of LXRs for the treatment of NAFLD, NASH, and other metabolic diseases.

CHAPTER 2. RESULTS

2.1 LXRs control inflammatory genes in Kupffer cells

Tissue-resident macrophages exhibit distinct roles that correspond to the functions of the tissue they reside in. As liver resident macrophages, Kupffer cells are involved in the regulation and signaling of inflammatory response genes through cytokine and chemokine release by sensing tissue injury. They are exposed to a wide range of biological signals, such as LPS and microbial products, as the liver is the first organ to receive blood from the intestine [33]. Therefore, Kupffer cells are integral to regulating the inflammatory response in the liver, but it is not fully known what factors contribute to the ability of Kupffer cells to modulate the injury and induce the wound healing response. LXRs are nuclear receptors that play a role in the inflammatory response program in different cell types and have been shown to decrease this inflammatory response during various inflammatory diseases, including atherosclerosis [24]. LXRs also have well-described anti-inflammatory functions in macrophages and have been speculated to be playing a key role in driving the Kupffer cell differentiation program [34,35]. Therefore, LXRs could be integral to the ability of Kupffer cells to signal in response to inflammatory mediators and it would be predicted that the loss of LXRs leads to an increased inflammatory response in Kupffer cells. This makes Kupffer cells an interesting cell type in which to study the roles of LXRs.

LXRα and LXRβ have different tissue and cell type distributions throughout the body. Kupffer cells and splenic red pulp macrophages are known to express high levels of LXRα compared to other macrophages [12,35,36]. Additionally, enhancer regions of Kupffer cells are enriched for DNA sequences that bind LXRs compared to other tissue macrophages [16]. Therefore, in order to investigate the relative abundance of LXRs in the liver, expression levels

were measured in the different cell types (Figure 2). RNA sequencing (RNA-seq) was performed on isolated hepatocytes, stellate cells, sinusoidal endothelial cells and Kupffer cells as well as whole liver tissue. The bar graph on the left shows the expression of LXRβ in each cell type and the bar graph on the right shows LXRα with expression levels in average transcripts per million.

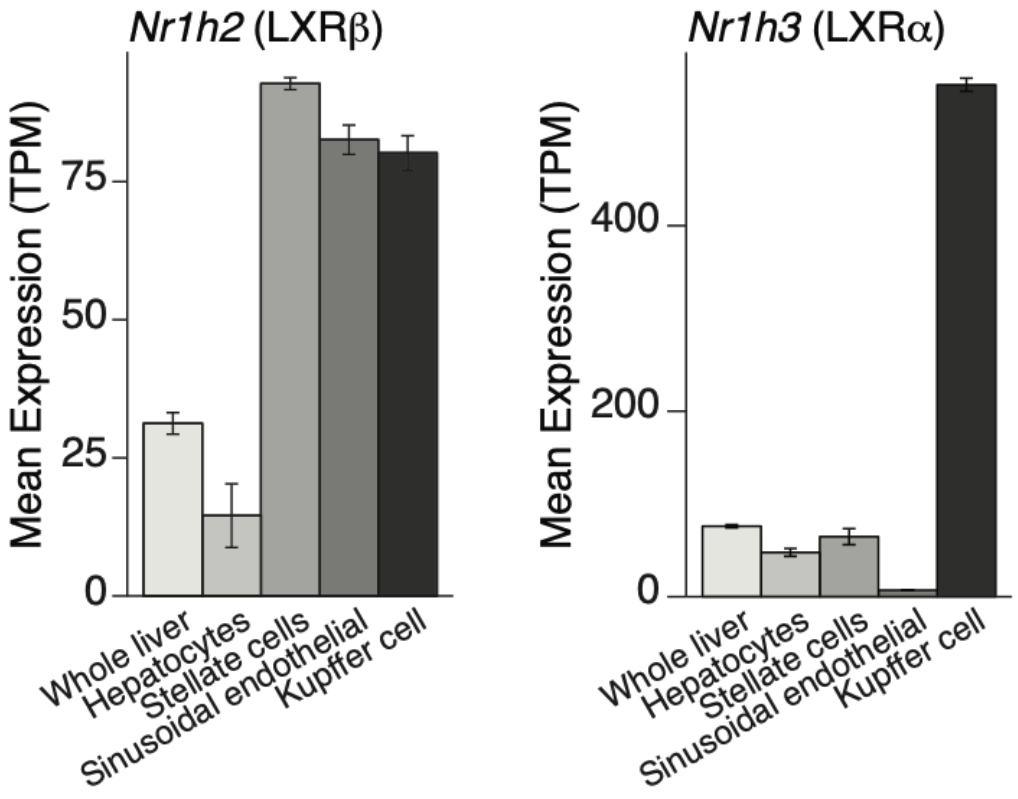

Figure 2. LXR alpha is highly expressed in Kupffer cells.
Average expression levels in transcripts per million (TPMs) of LXRα (*Nr1h3*) and LXRβ (*Nr1h2*) from RNA-seq data in the various cell types of the liver. LXRα is most highly expressed in Kupffer cells, and therefore is likely to play a role in Kupffer cell function and may be an important lineage determining factor.

This data confirms the findings that LXRα is highly expressed in Kupffer cells, and further reveals that Kupffer cells express at minimum 7-fold more LXRα transcripts per million than whole liver, hepatocytes, hepatic stellate cells, or liver sinusoidal endothelial cells. These expression differences reveal that Kupffer cells are the dominant hepatic source for LXRα

expression, whereas LXRβ is expressed similarly between multiple liver cell types. In addition, LXRα has a six-fold higher expression level in Kupffer cells than LXRβ, therefore it may be playing a more profound role in Kupffer cells. LXRα is very lowly expressed in the other cell types of the liver, illustrating that LXRα may be important for Kupffer cell differentiation and contain instructions for the unique functions of Kupffer cell.

In order to assess the role of LXR expression in the maintenance of homeostatic gene expression programs, Kupffer cells from mice in which both LXRα and LXRβ were absent throughout all tissues were isolated using fluorescence activated cell sorting (FACS). The cell sorting strategy (Figure 3) includes a series of gates used to remove debris and unwanted cells to isolate a pure population of Kupffer cells. These mice will be denoted as full body double knockout (DKO) mice. RNA-seq was performed on these Kupffer cells, as well as on wild type Kupffer cells isolated using the same method (Figure 4).

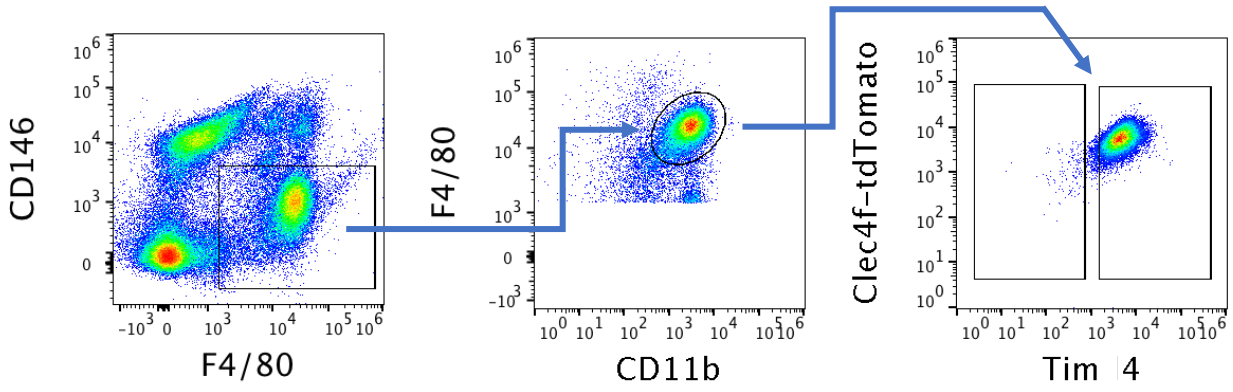

Figure 3. Kupffer cell sorting strategy.
Fluorescence activated cell sorting strategy to isolate a pure population of Kupffer cells. Cells are first gated on F4/80 and CD146 to isolate macrophages and remove endothelial cells, followed by live cell and singlet selection gates (not shown). Lastly, cells are gated on Tim4, which is a cell surface marker.

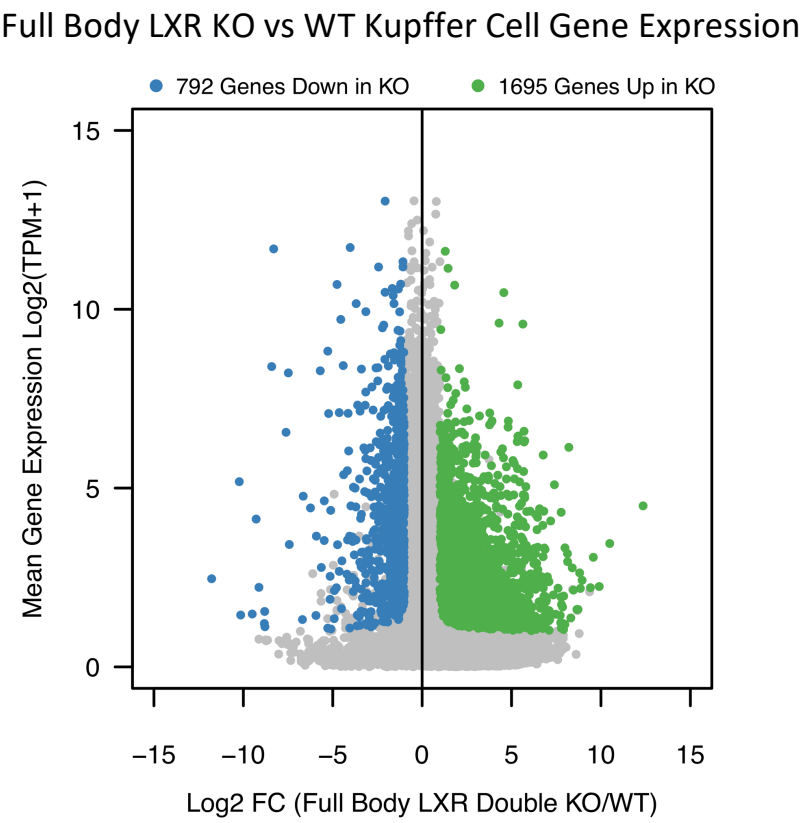

Figure 4. LXRs control inflammatory genes in Kupffer cells.
RNA-seq comparison of Tim4^{High} Kupffer cells from full body LXRα and LXRβ KO or WT mice. Plot of fold change versus mean TPM values, blue dots are downregulated in the LXR DKO mouse (TPM >2, FDR <.05, and fold change <2), green dots are upregulated in the LXR DKO mouse (TPM >2, FDR <.05, and fold change >2).

There are over 2,000 differentially expressed genes in full body DKO mice compared to wild type, revealing that LXRs are required to maintain normal expression of these genes in Kupffer cells. The upregulated genes consist of inflammatory pathway genes, including many from the wounding response and inflammatory response revealed using the gene ontology analysis tool Metascape (Table 1). Important genes in particular that are upregulated include *Il1r*, which is involved in many cytokine-induced immune and inflammatory responses; *Ccr2*, which is a chemokine that mediates monocyte chemotaxis; *Ccl5*, which is also in the chemokine family

and plays a role in immunoregulatory and inflammatory processes; *Saa3*, which induces inflammatory cytokines; and *Cx3cr1*, which is an adhesive and migratory gene usually associated with monocytes. The upregulation of these genes illustrates a higher amount of inflammation when LXRs are absent, indicating that LXRs play an anti-inflammatory role in Kupffer cells. *Cx3cr1* and *Ccr2* are particularly interesting because they are chemokine receptors that play a role in monocyte recruitment into the liver. This suggests that these cells may be monocyte-derived, rather than tissue-resident. This increased expression may translate to the cell being less "Kupffer cell-like" and more "monocyte-like." This reveals there is heterogeneity in the Kupffer cell population and that LXRs are not only involved in inflammatory pathways in Kupffer cells, but also important for maintaining the Kupffer cell properties.

Table 1. Gene ontology analysis of upregulated genes in LXR DKO mice.
This table shows the relevant GO terms using the gene ontology tool called metascape. Values are log q-values using upregulated genes from figure 3. This shows an increase in the inflammatory pathway when LXRs are absent.

GO Term	Log (q-value)	Associated Genes
Wounding response	-33.30638362	*Ccr2, Col3a1, Col5a1, Col1a1, Mmp12*
MAPK cascade	-27.80958209	*Adam8, Ccr1, Ccr3, Ccl5, Cx3cl1*
Inflammatory response	-17.31345707	*Il1r1, Ccr2, Socs3, Ccl5, Cx3cr1, Pparg, Adam8, Saa3*
Regulation of inflammatory response	-10.02372511	*Socs3, Ccr2, Il1r1, Pparg, Ccl5*

This section has revealed that LXRα is highly expressed in Kupffer cells, making it an interesting topic for further investigation. In addition, LXRs play a role in regulating the inflammatory response, as LXR DKO mice show an increase in inflammatory pathway genes, including some monocyte specific genes. This has important relevance to NASH, as an inflammatory disease that may be influenced by LXRs. There is an infiltration of monocytes during NASH which is correlated with disease severity and length of disease progression. The activities of recruited hepatic myeloid cells, as well as myeloid cell driven inflammation in the liver make significant contributions to metabolic and inflammatory alterations occurring during NAFLD/NASH. The field is currently debating the origin of myeloid cells, but it is thought that infiltrating monocytes can gain some tissue resident cell function and may start to acquire Kupffer cell markers. Therefore, it will be interesting to investigate LXR deficient mice in the context of NASH, as well as the gene expression alterations of a Kupffer cell specific knockout mice.

2.2 Cell-specific KO reveals importance of LXRs at steady state and during disease

The experiments presented in the previous section were performed on mice that were lacking LXRs throughout every tissue in the body, which may be a confounding factor to the roles of LXRs in Kupffer cells due to the dysregulation of cholesterol throughout the entire body. Therefore, in order to investigate a more specific function of LXR target genes in Kupffer cells, two specific types of Cre were used. For many tissue resident macrophages, including Kupffer cells, lysozyme 2 (Lyz2) driven Cre expression effectively mediates target gene excision and has been widely used by the research community [37]. This Cre mouse was crossed to $LXR\alpha^{Fl/Fl}$ $LXR\beta^{Fl/Fl}$ mice to create a strain that will be denoted Lyz2 Cre $LXR\alpha^{Fl/Fl}$ $LXR\beta^{Fl/Fl}$ mice. However, Lyz2 is expressed by other tissue resident macrophages, including circulating

monocytes and neutrophils, which leads to genetic excision of gene targets throughout the myeloid compartment. This model is useful; however, a more specific mouse in which LXRs are deleted only from Kupffer cells would help determine the Kupffer cell specific LXR dependent gene expression programs during NAFLD/NASH progression in mice. Clec4f expression occurs only in Kupffer cells and is not expressed by other tissue resident macrophages or circulating myeloid cells [16,38]. Thus, a Kupffer cell specific Cre strain will help provide a way to elucidate the role of target genes in Kupffer cells. Therefore, LXR$\alpha^{Fl/Fl}$ LXR$\beta^{Fl/Fl}$ mice were also crossed to this Kupffer cell specific Cre strain, which will be denoted Clec4f LXR$\alpha^{Fl/Fl}$ LXR$\beta^{Fl/Fl}$ mice.

Using the sorting strategy described previously (Figure 3), flow cytometry and fluorescence automated cell sorting were performed with both Lyz2 Cre LXR$\alpha^{Fl/Fl}$ LXR$\beta^{Fl/Fl}$ mice and Clec4f LXR$\alpha^{Fl/Fl}$ LXR$\beta^{Fl/Fl}$ mice and compared to a Cre negative controls (Figure 5).

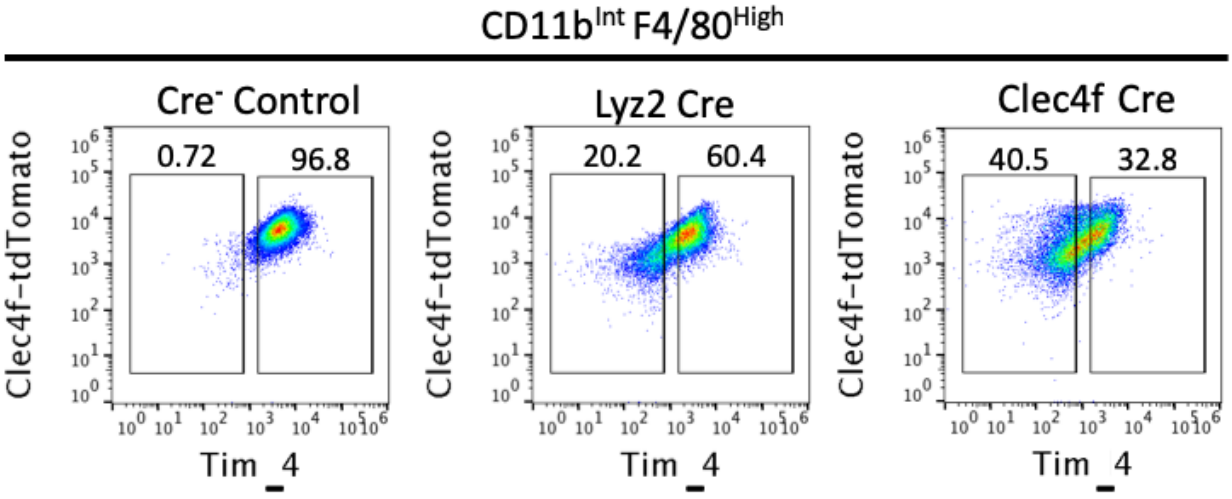

Figure 5. LXRs are required for the maintenance of Tim4 expressing Kupffer cells. Flow cytometry plots of Kupffer cells from LXR$\alpha^{Fl/Fl}$ LXR$\beta^{Fl/Fl}$ of the indicated Cre type. We see an increased number of Tim4^{neg} macrophages when LXRs are absent, as well as a larger group of this population in the Clec4f Cre LXR knockout mice. Tim4^{neg} cells accumulate during fatty liver disease.

The last marker that the Kupffer cells are gated on is Tim4, which is a cell surface marker present on Kupffer cells. This is an interesting marker because it is thought that $Tim4^{pos}$ cells are resident or long term Kupffer cells and $Tim4^{neg}$ cells are monocyte-like cells that infiltrate the tissue during inflammatory diseases [39,40]. The flow cytometry plot on the left is a Cre negative control and the plots on the right and center are the two types of floxed mice. We can see from these plots that the number of $Tim4^{neg}$ cells increase when LXRs are absent, and the number of $Tim4^{pos}$ cells decrease. This means that LXRs are necessary for the maintenance of Tim4 expressing Kupffer cells. As mentioned, $Tim4^{neg}$ Kupffer cells are correlated with inflammatory disease, so it is very interesting that the population of $Tim4^{neg}$ follow the trend of the effects of mice with NASH from a NASH-inducing diet. This reveals the importance of LXRs to maintaining a homeostatic cell population of Kupffer cells in the liver.

In order to investigate the transcriptional alterations underlying this change in the Kupffer cell population, RNA-seq was performed on the $Tim4^{pos}$ cells from the flow plots shown above. The expression levels of LXRα and LXRβ were examined to see the efficiency of the Cre systems for both Lyz2 Cre and Clec4f Cre compared to a Cre negative control (Figure 6).

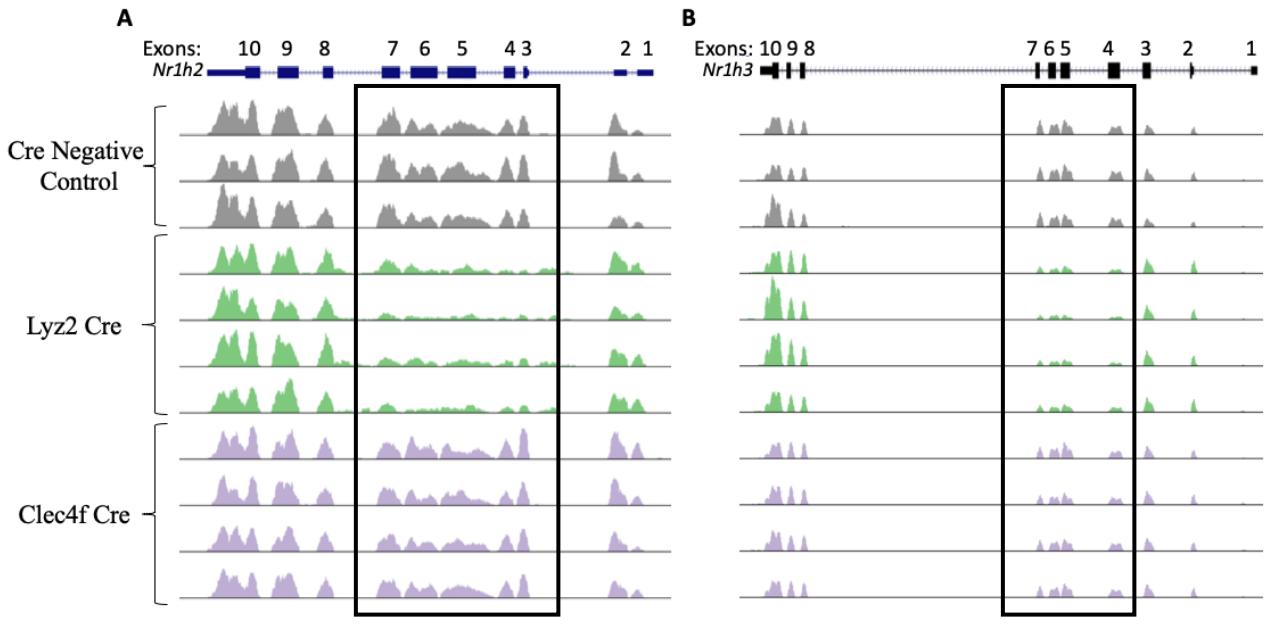

Figure 6. Efficiency of Lyz2 Cre and Clec4f Cre in LXRα$^{Fl/Fl}$ LXRβ$^{Fl/Fl}$ mice.
UCSC Genome Browser tracks showing mRNA expression of (A) *Nr1h2* (LXRβ) and (B) *Nr1h3* (LXRα) at each exon. Black boxes surround exons expected to be lost. Each block of colors are replicates. Grey plots are of Cre negative controls, green are Lyz2 Cre, and purple are Clec4f Cre. Lyz2 Cre appears to have a better knockout efficiency.

The browser tracks reveal some unexpected findings, as there is not a complete gene knockout for either Cre type. There is, however, a reduction in expression in the Lyz2 Cre floxed mouse (green), illustrated by the decreased peaks in the exons surrounded by the black boxes for both LXRs compared to the Cre negative control (grey). This means that LXRα and LXRβ are effectively recombined; however the recombination efficiency is not one hundred percent since there is a low level of expression still present. This could be due to the previously described efficiency of Lyz2 Cre having some variation in recombination [37]. However, as previously mentioned LXRs may be an important cell lineage determining factor for Kupffer cells [35]. Recent studies have suggested a pro-survival role of LXRs in Kupffer cells and splenic macrophages [35,36]. This could mean that LXRs are necessary for Kupffer cell survival and

that the cells are unable to survive once LXRs are inactivated, leading to cell death. Further, as a result of the loss of Kupffer cells, there is an infiltration of monocytes that could be contaminating the Tim4^{pos} population of Kupffer cells. The Clec4f Cre mouse does not appear to have a lower expression of the LXR alpha or beta exons, which reveals other problems with sorting the Clec4f Cre LXR$\alpha^{Fl/Fl}$ LXR$\beta^{Fl/Fl}$ mice.

There are a few hypotheses that may explain the lack of Clec4f Cre recombination for LXRs in the sorted population of Tim4^{pos} cells. The Clec4f Cre mouse has an engineered allele driven by an internal ribosome entry site encoding a polypeptide for Cre and a T2A cleavable nuclear localized fluorescent tdTomato reporter. This provides a bright marker of Kupffer cells that can be readily detected by flow cytometry. Therefore, the proper sorting strategy must be employed in order to get optimal tdTomato expression, so further experiments were sorted with a FACS machine that has a larger number of channels in order to optimize expression and separation of populations. Another speculation is that the lack of LXRs could result in reduced embryonic Kupffer cell survival rate and cause the replacement by monocyte derived cells. Clec4f is expressed after the embryo starts to develop, which means tdTomato will label the Kupffer cells when they begin expressing Clec4f Cre. There is the possibility that once the cells begin expressing Cre, they will flox the LXRs and then not be able to survive. Therefore, there are various obstacles at play here in aiming to understand the contribution of LXRs to resident Tim4^{pos} Kupffer cells.

Understanding of the composition of the macrophage heterogeneity in the liver, including Kupffer cells and monocyte-derived macrophages, during non-homeostatic conditions, such as inflammation and injury is not fully understood. There is a lack of specific markers to distinguish between the different cell types, so the alteration during NASH pathogenesis is not fully known

[40]. Therefore, to investigate this principle in a disease state, Lyz2 Cre and Clec4f Cre LXRα$^{Fl/Fl}$ LXRβ$^{Fl/Fl}$ mice were placed on a NASH-inducing high fat and cholesterol diet for 20 weeks. The Kupffer cells were then sorted and flow plots were compared to negative controls.

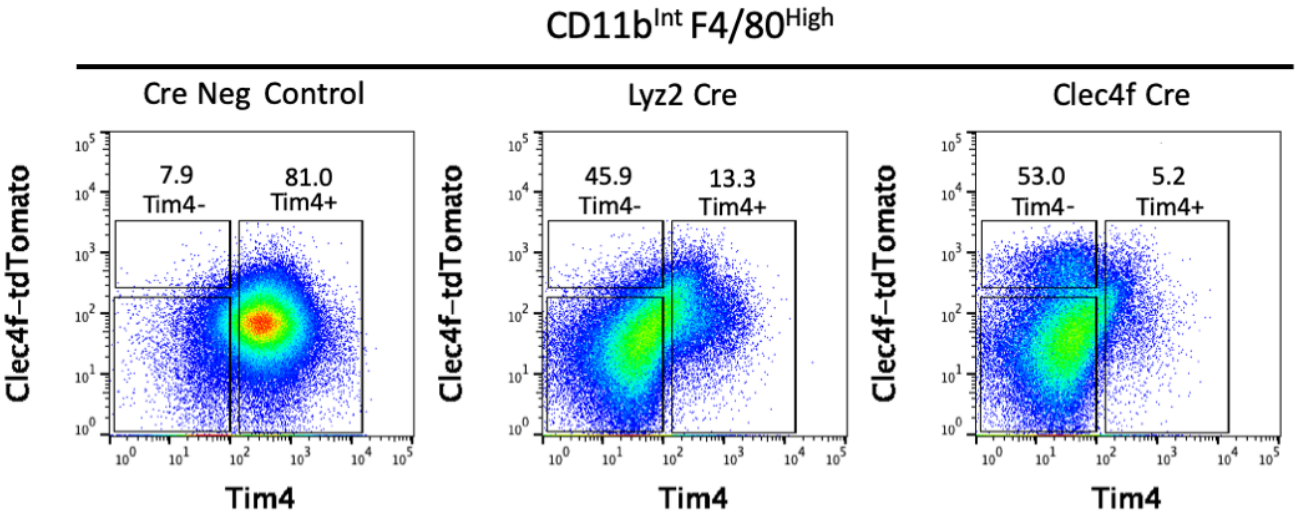

Figure 7. LXRs are important for maintenance of Tim4^{Pos} Kupffer cells during NASH. Flow plots of Tim4 expression on the x-axis and Clec4f-tdTomato expression on the y-axis of Kupffer cells lacking LXRs with both Cre types compared to control. Numbers represent percentages of cells in each population. There is an increase in Tim4^{neg} cells when LXRα$^{Fl/Fl}$ LXRβ$^{Fl/Fl}$ mice are placed in the NASH diet.

These flow plots reveal interesting speculations about LXRs and NASH. First, they show that the Tim4^{neg} Kupffer cell population drastically increases when LXR-null mice have developed NASH compared to Cre negative control mice that have also developed NASH. This suggests that when LXRs are lost there are more infiltrating cells which is correlated with higher disease severity. Therefore, LXRs may play an integral role in regulating Kupffer cells during NASH. When LXRs are absent the disease state may be exacerbated, revealing that activation of LXRs could be beneficial during NASH. In these flow plots, we are able to see a separation of tdTomato on the y-axis, an improvement from the steady state FACS in figure 5. However, there

are not a large number of tdTomato positive cells. This is a possible explanation for the lack of knockout efficiency of Clec4f LXRα$^{Fl/Fl}$ LXRβ$^{Fl/Fl}$ mice shown in figure 6. The tdTomato positive cells have a very small population, making genomics assays difficult.

One hypothesis is that the loss of LXRα/β signaling leads to apoptosis of Kupffer cells in NASH, thereby reducing the population of Tim4^{pos} Kupffer cells. This could provide an open niche that enables the recruitment of monocytic precursors. Further, there is the possibiliy that the Tim4^{pos} tdTomato negative population have somehow escaped Cre expression and recombination. Recent studies have demonstrated that Tim4^{neg} cells are consistent with monocytes, rather than embryonic origin among liver F4/80^{Hi} cells, so these findings suggest that monocyte derived macrophages may contribute to the pool of what have previously been considered "resident" cells during NASH [39]. These distinctions are important when interpreting the findings of genomics assays, including RNA-seq data, because it is difficult to tell if the observed effects represent cell-intrinsic changes of the populations or are due to the recruitment of a separate group of disease responsive cells. However, this sorting strategy allowed for the separation of tdTomato, so follow-up experiments can be performed that aim to collect the fully knocked out populations to investigate the alteration in gene expression in order to reveal more about this complex heterogeneity during NASH when LXRs are absent.

From this group of mice; however, liver samples were collected for histology and stained with a trichrome stain that stains for fibrosis and reveals steatosis and inflammation (Figure 8).

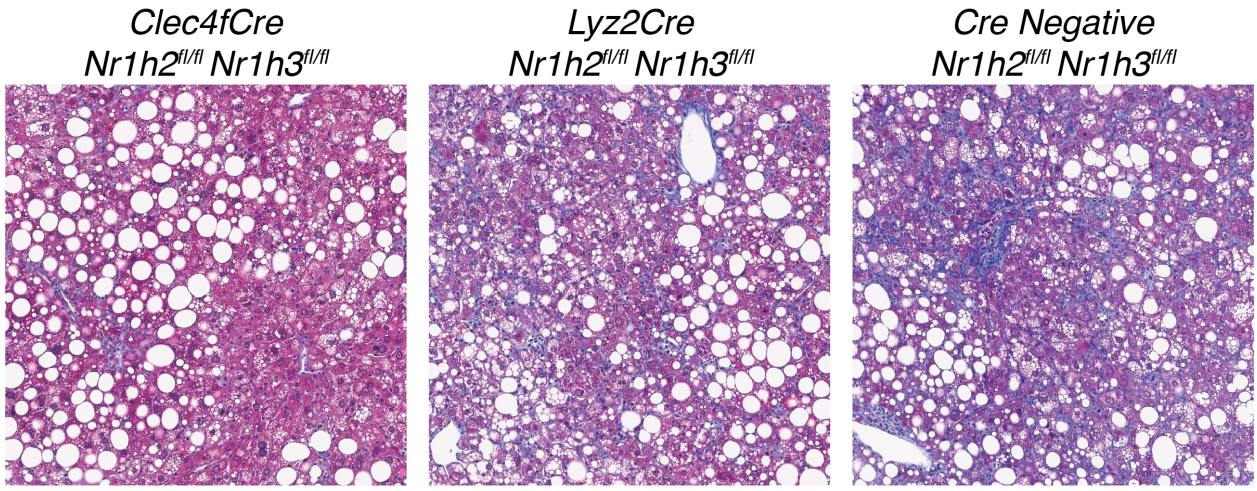

Figure 8. LXRs may be anti-inflammatory and beneficial during NASH.
Liver sections of Lyz2 Cre and Clec4f Cre LXR$\alpha^{Fl/Fl}$ LXR$\beta^{Fl/Fl}$ stained with trichrome in order to visualize steatosis, inflammation and fibrosis. Collagen is stained blue, white spots are lipid droplets called steatosis, and areas of dense nuclei depict inflammation.

Further quantification by a pathologist is necessary to draw more certain conclusions; however, as pilot data, a few speculations can be made. Steatosis is defined by the size and abundance of lipid droplets. The LXR knockout mice may have more steatosis, however it needs to be quantified. This could mean that LXRs are beneficial during NASH because when they are absent there is a more severe NASH pathology. However, there may be less fibrosis in the LXR knockout mice. More replicates and professional quantification are necessary to draw definite conclusions, but this finding supports the flow cytometry data descried previously and once more replicates are analyzed could suggest that activation of LXRs may be beneficial during NASH.

2.3 Activation of LXRs by DMHCA is beneficial during NASH

As shown in the previous sections, the loss of LXRs results in an increase in inflammatory gene expression, infiltration of monocytic cells, and worsened pathophysiology during NASH. This has revealed the importance of LXRs to maintaining homeostasis at the

steady state as well as during NASH, especially in Kupffer cells that are involved in regulating inflammation and injury in the liver. The activation of LXRs have been shown to decrease downstream pro-inflammatory gene expression and suppress inflammatory response genes during disease [23,24]. Therefore, as regulators of cholesterol and lipid homeostasis, their activation may be beneficial at the steady state and particularly during the progression of NASH. LXRs signal changes in gene expression when a ligand binds to the LXR binding domain followed by a co-activator binding to the complex [19]. LXRs were originally called orphan receptors, meaning that their binding partners were unknown at the time they were discovered [23]. Since the finding that cholesterol derivatives and oxysterols are the ligands of LXRs, scientists have been trying to synthesize molecules that are able to selectively bind and activate them [30]. Many synthetic mimetics have been created that activate LXRs, however there is a major problem with these molecules. As discussed earlier, SREBP, which is involved in the fatty acid synthesis pathway is regulated by LXRs. Therefore, most synthetic LXR agonists have been shown to raise serum triglycerides and cause hepatic steatosis in addition to promoting cholesterol export, making them poor drug candidates [27,29,30].

As mentioned in the introduction, desmosterol is the primary LXR ligand present in macrophage foam cells, which suppresses SREBP processing at the endoplasmic reticulum and does not drive fatty acid biosynthesis [24]. Therefore, a molecule similar in structure to this natural ligand would be most likely to function in the same way. One molecule called DMHCA has a very similar structure to desmosterol, making it a promising candidate for selectively activating LXRs. One of the first papers to study synthetic ligands, such as GW3965, T0901317, and DMHCA, tested the different molecules both orally and by intraperitoneal injections. They found by qPCR in mouse liver, that DMHCA was the only molecule able to turn on the

cholesterol efflux gene *Abca1* without simultaneously upregulating *Srebp1* and fatty acid synthesis. This revealed that synthetic ligands have an effect on the liver at the steady state, prompting scientists to investigate activation of LXRs during disease. For example, the LXR agonist GW3965 was found to have strong antiatherogenic activity in LDLR KO and ApoE KO mice [30]. Another study found that the treatment of mice with a molecule called SR9238 significantly reduced the severity of hepatic steatosis and even decreased inflammation and fibrosis of mice on a NASH model diet [41]. Since these findings, many studies have been performed with various synthetic mimetics with the aim to promote the beneficial properties of LXR activation, including cholesterol efflux in macrophages, bile acid synthesis in the liver, and the inhibition of intestinal cholesterol absorption. Surprisingly, there are very few studies using DMHCA to activate macrophages during disease, though activation of LXRs in Kupffer cells may alter gene expression and modulate liver disease.

In order to investigate the effects of DMHCA in Kupffer cells, DMHCA was injected intraperitoneally at a dose of 50 mg/kg into C57BL/6J mice and mice were harvested after 12 hours. The livers were dissected and Kupffer cells were sorted with FACS. RNA-seq was performed on the sorted Kupffer cells and gene expression levels were examined (Figure 9). This plot shows the fold change expression in log values of DMHCA treatment compared to vehicle.

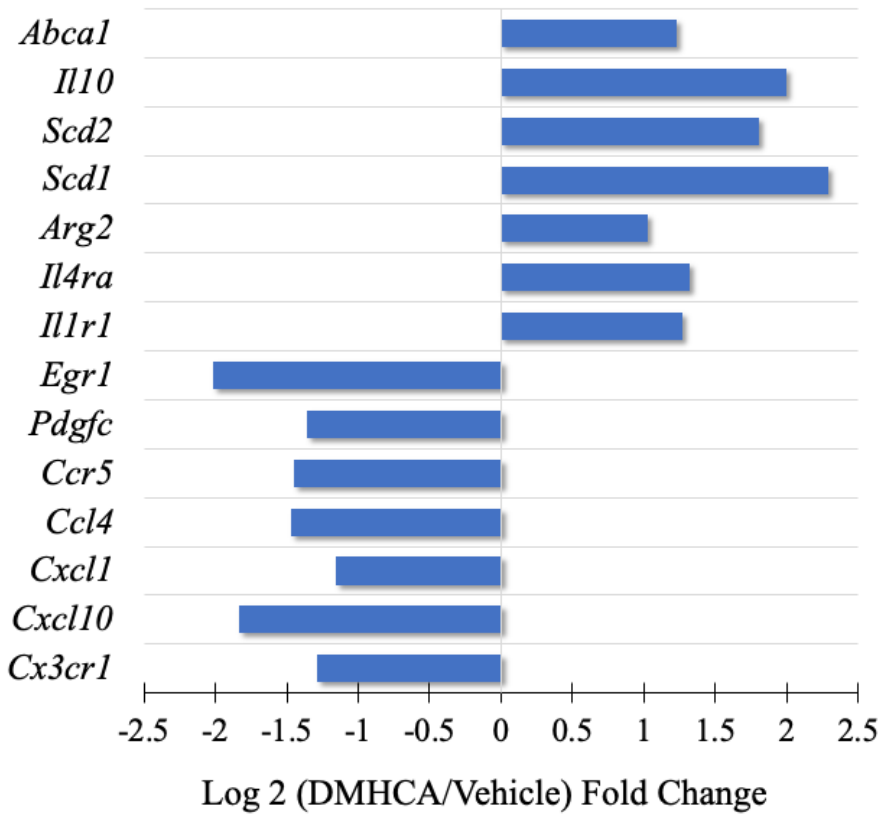

Figure 9. DMHCA treatment regulates inflammatory gene expression in Kupffer cells. Mice were treated with DMHCA by IP injection (50 mg/kg) for 12 hours. RNA sequencing was performed on FACS purified Kupffer cells. Data represents fold change values calculated using DESeq2 comparing DMHCA and vehicle treatment. Genes listed include LXR target genes and inflammatory response genes.

This data reveals an alteration of gene expression by DMHCA modulated activation of LXRs. There is an increase in LXR target genes, such as *Abca1*, which suggests that cholesterol efflux is increased. In addition, there is an increase in anti-inflammatory genes, such as *Scd1* and *Scd2*, which are stearoyl-CoA desaturase enzymes that function in lipid biosynthesis and are thought to be beneficial and have anti-inflammatory properties. *Il10* is also an anti-inflammatory gene that is increased with DMHCA treatment. Inflammatory genes appear to decrease with DMHCA treatment, such as *Ccr5* and *Cxcl10*, which are involved in the pro-inflammatory response. This illustrates that DMHCA is able to activate LXRs in Kupffer cells, as well as turn on downstream

target genes that decrease inflammation. *Srepb1* is not significantly increased, as it has a log 2-fold change below the cutoff of 1 (not shown on plot), therefore DMHCA is able to turn on *Abca1* without simultaneously activating fatty acid synthesis. In summary, activation of LXRs by DMHCA at the steady state appears to decrease inflammatory gene expression programs in Kupffer cells.

I next sought to study the effect of LXR activation during disease, as DMHCA modulated activation at the steady state revealed promising insight that this molecule may have anti-inflammatory properties during NASH. Oral-based pharmaceuticals are more commercially viable than an injection-based delivery method. Therefore, in order to therapeutically target LXR signaling in NASH, mice were treated with oral DMHCA supplemented in a NASH-model diet (Figure 10). This study is significant, as it could potentially identify a new class of therapeutics for the treatment and prevention of NASH. Further, this study aims to gather new mechanistic insight into the role of LXR signaling and cholesterol homeostasis tissues during NASH pathogenesis, along with a new therapeutic use of LXR agonists.

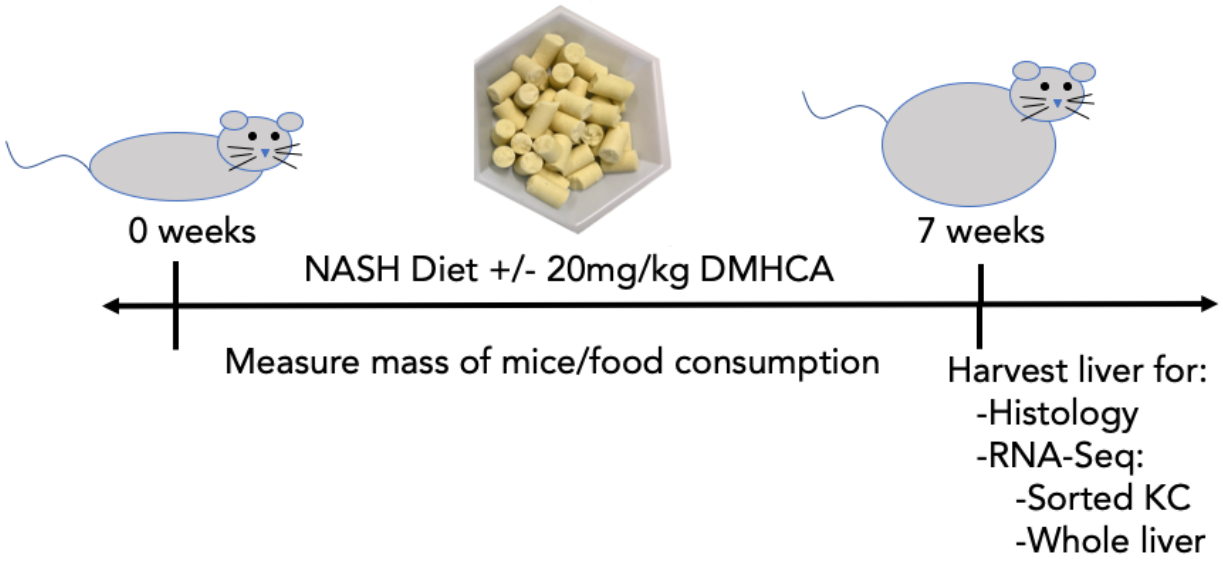

Figure 10. NASH model diet supplemented with DMHCA experimental design. Schematic showing the experimental design for a high fat NASH model diet with added DMHCA to test whether oral DMHCA prevents early states of NAFLD development in mice. Mice were singly housed and fed a NASH-model diet composed of 40 kcal% fat and 2% cholesterol with or without 20 mg/kg/day DMHCA. Mice were weighed and fed weekly for 6 weeks, and were then sacrificed and liver tissue was collected for histology and Kupffer cells were sorted for RNA-seq.

Mice were fed a NASH-inducing diet with or without 20 mg/kg/day DMHCA supplemented into the food. This NASH-inducing diet has a high percentage of fat and cholesterol and is used throughout the research community. DMHCA is a mimetic that does not cause hypertriglyceridemia or fatty liver, making it a strong candidate to study its effects on the development of NASH. The unwanted side-effects of other LXR mimetics would contribute to the NASH phenotype rather than decrease NASH severity. LXRs are expressed in hepatocytes, macrophages, and enterocytes and LXR agonism has been shown to induce reverse cholesterol transport and cholesterol export. I hypothesize that DMHCA treatment will lower free hepatic cholesterol and decrease inflammation, fibrosis, and steatosis in mice fed a NASH-model diet. The mice were singly housed so weight and food consumption could be measured weekly. Mice

from both groups displayed equivalent food intake while mice fed DMHCA trended towards lower weight compared to mice fed the NASH diet without DMHCA (Figure 11).

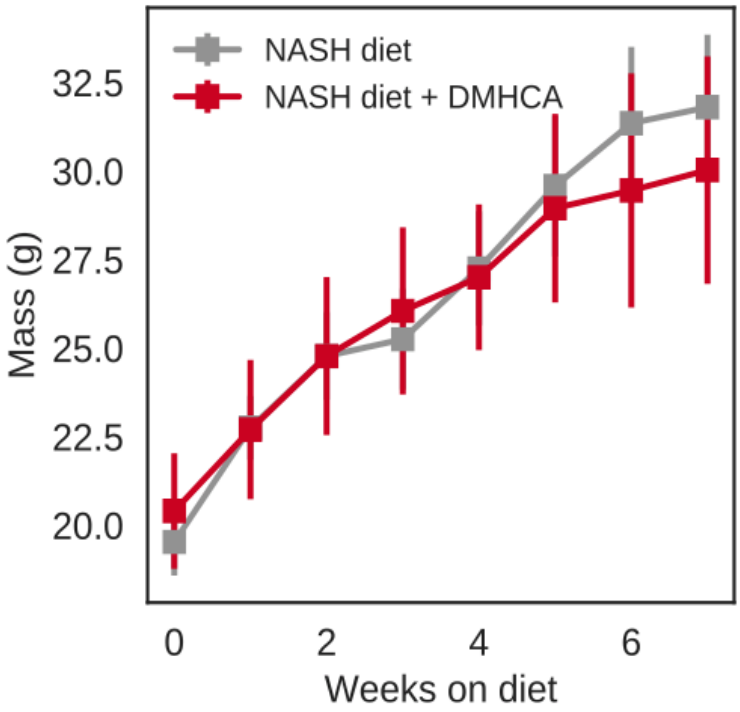

Figure 11. Average mass of NASH-fed mice with or without DMHCA.
Plot shows average weight curves of mice on the NASH diet with supplemented DMHCA (red line) compared to the NASH diet (Gray line) over 7 weeks. Mice on the NASH plus DMHCA diet appear to trend towards a lower weight than the NASH without DMCHA diet.

The sample size for this study was 4 mice per group due to it being a pilot study; however, it appears that DMHCA may have a weight phenotype. There could be many underlying causes for this lower trend of average mass for the DMHCA treated mice, which will be discussed further at the gene expression level later in this section. However, one explanation is that the activation of LXRs could be helping to cause cholesterol efflux. A dysregulated lipid profile is associated with obesity-linked metabolic syndrome. This disease includes hypertriglyceridemia, as well as low levels of plasma high-density lipoprotein (HDL)

cholesterol. HDL cholesterol is thought to initiate reverse cholesterol transport, and therefore lower levels in dyslipidemic patients may cause impaired RCT [42]. The activation of LXRs has been shown to increase RCT, therefore LXR activity may be compensating for the loss of the ability to perform reverse cholesterol transport and cholesterol efflux. This may contribute to a lower body weight when LXRs are activated by DMHCA and have functioning RCT. Additional replicates and a longer study are necessary to confirm this preliminary finding.

After 7 weeks on the NASH or NASH with DMHCA diet, mice were sacrificed, and a portion of their liver tissue was collected and sectioned for histological analysis (Figure 12).

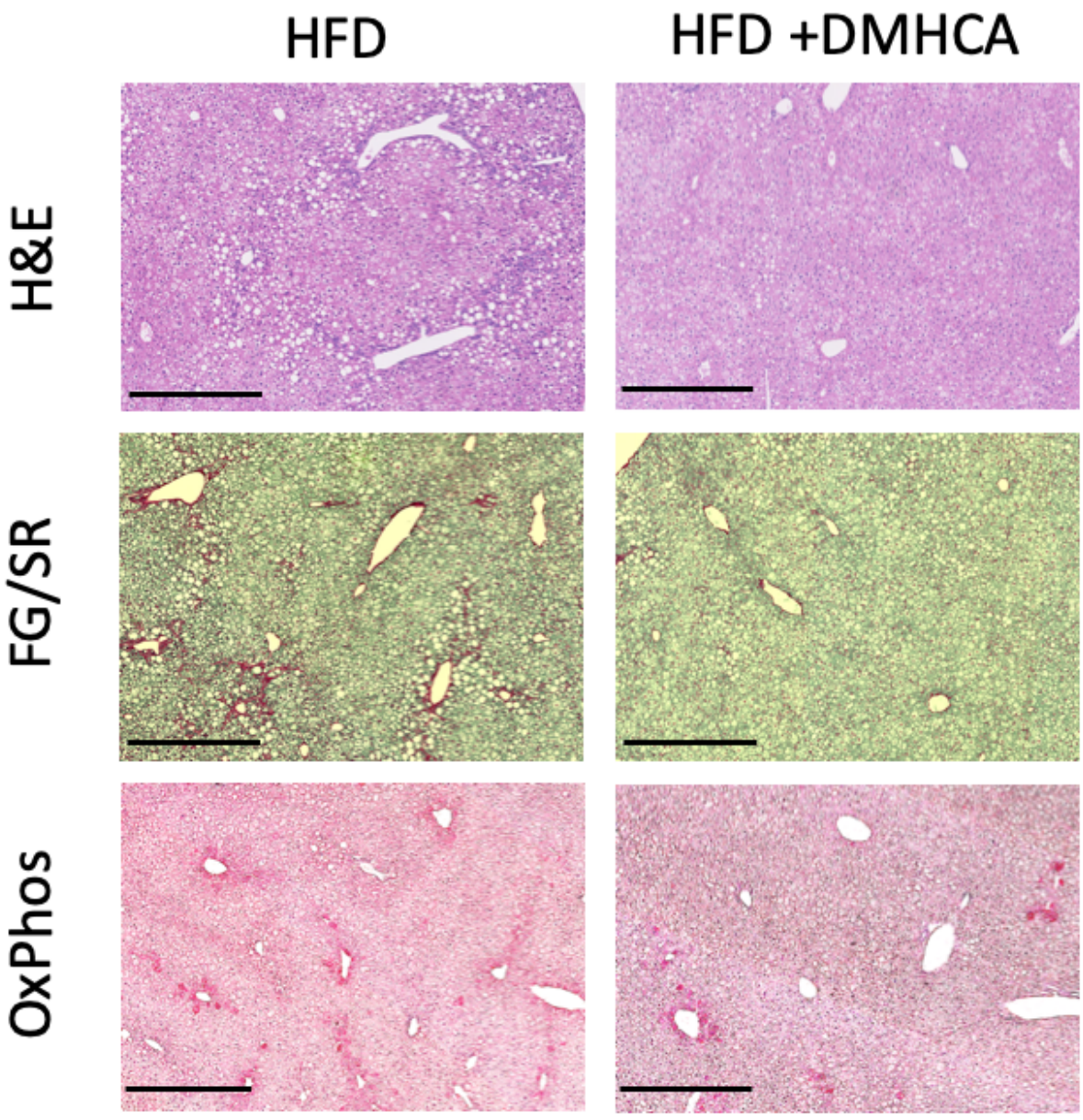

Figure 12. DMHCA treatment improves liver disease pathology during NASH. Paraffin embedded sections stained with (from top to bottom) hematoxylin and eosin (H&E), sirius red with fast green background (FG/SR), or oxidized phospholipid sites using an E06 antibody (OxPhos). Scale bar denotes 500 microns. Mice were fed a 40% high fat diet for 7 weeks. The treatment group was supplemented with DMHCA mixed in the food. This preliminary histological data suggests a protective phenotype from DMHCA.

NASH usually develops around 10 weeks and becomes increasingly severe at 20-30 weeks, so the timepoint chosen shows the early stages of NASH. As discussed in the introduction, histological analysis allows for the visualization of the pathophysiologic progression of NASH by revealing the characteristics of this disease including fibrosis, steatosis, and lobular inflammation. Therefore, three different types of stains were used to visualize these characteristics in Figure 12. Though the sample size is small, and a trained pathologist is necessary to accurately quantify the slides, some qualitative conclusions can be drawn. These histological results show a qualitative decrease in hepatic steatosis and inflammation in mice treated with DMHCA. The top panel stained with hematoxylin and eosin, allows for the visualization of inflammation by staining nuclei. Areas with a high density of cell nuclei reveal inflammation due to the infiltration of many inflammatory cells such as monocytes and neutrophils, called lobular inflammation. H&E also allows for the visualization of steatosis, characterized by unstained fat droplets. It appears that there is less infiltration and steatosis in mice treated with DMHCA, illustrated by a decreased amount and size of lipid droplets and no areas of dense nuclei.

The middle row shows a sirius red stain with a fast green background that stains for fibrosis, which is scar tissue that arise from collagen deposits during liver injury. The DMHCA treated group appears to have less fibrosis than the untreated group. Lastly, an oxidized phospholipid marker stain was used in the bottom panel, which consists of an E06 antibody that labels phospholipids [43]. DMHCA treated mice appeared to accumulate less E06 antibody staining, which is depicted by the areas of dark pink staining, than control mice, suggesting less severe metabolic disease. It is known that during inflammatory diseases, there in an increase in oxidized phospholipids which contribute to the proinflammatory environment. Overall, it appears

that treatment with the LXR agonist DMHCA causes a phenotypic change in the liver environment during NASH resulting in a decrease in the indicators of NASH. This is a significant finding, as there is no current therapeutic agent to treat NASH. This study has revealed that selective LXR agonism alters the early stages of NASH and may be able to prevent its progression to more severe forms such as cirrhosis. This also means that LXRs may be dysregulated during NASH which could be leading to decreased signaling and subsequently the progression of the disease. This could be due to the altered environment and the infiltration of Tim4 negative inflammatory cells.

This protective result reveals changes at the phenotypic level, so next RNA-seq was performed on liver pieces from these mice to asses genomic changes. Whole liver gene expression showed modest differences between DMHCA treated and untreated mice (Figure 13).

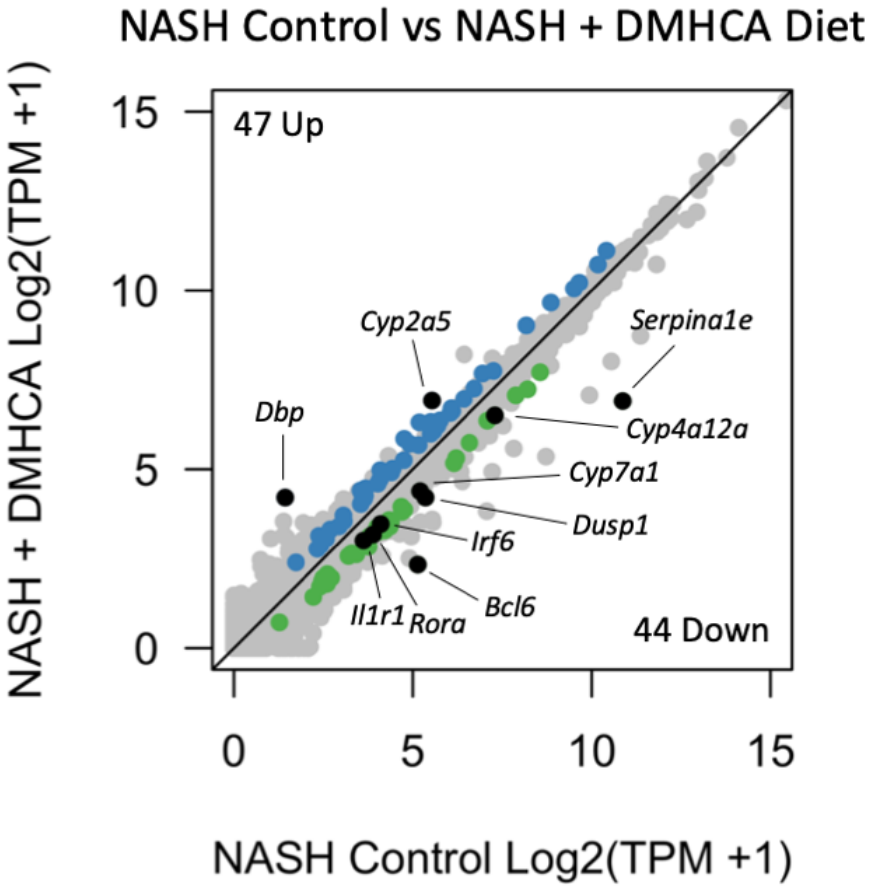

Figure 13. DMHCA diet has minimal effects on whole liver gene expression.
RNA-seq comparison of whole liver tissue from mice fed a NASH model diet with and without DMHCA for 7 weeks. Each treatment group consisted of 3 mice. Plot of TPM values; blue dots depict upregulated genes (FDR<0.05, FC >1.5); green dots depict downregulated genes (FDR <0.05, FC >1.5). Black dots depict labeled genes of interest. DMHCA treatment appears to have a minimal effect on the liver.

The histological data showed alterations in the characteristic phenotype of NASH, but surprisingly, differential gene expression analysis reveals a minimal change in gene levels in the liver. DMHCA did not stimulate hepatic transcription of the LXR target genes *Abca1, Abcg1, Abcg5, Abcg8, or Srebf1*, which is in agreement with prior studies showing that intraperitoneally administered DMHCA does not activate hepatic LXRs [30]. Interestingly, DMHCA treatment was associated with a nearly 2-fold decrease in expression of the LXR target gene *Cyp7a1*. This

could suggest that DMHCA encourages cholesterol excretion outside of the liver, possibly leading to lower hepatic free cholesterol and decreased expression of cholesterol exporting genes in the liver. This is an unusual result, as significant alteration at the gene expression level would be expected from the major histological changes. However, these studies were performed at an early phase of NASH progression, so a follow-up experiment assessing a long-term model of NASH pathogenesis may reveal genomic changes. If DMHCA is able to phenotypically alter the liver at this stage of disease, it could have drastic effects on the progression of this disease to a much later timepoint.

This section has revealed that DMHCA has the potential to be a promising therapeutic for treating the development and progression of NASH. Activation of LXRs decreased inflammatory genes at the steady state in Kupffer cells and led to a major phenotypic change in the liver during NASH. Administering DMHCA orally was protective against NASH development, which is exciting due to the lack of studies performing oral dosage during liver disease. Kupffer cells are responsible for initiating proinflammatory response programs in the liver, so LXRs may be essential to regulating this response. Though there was a lack of gene expression alteration in the liver, DMHCA was able to improve the characteristics of NASH. Therefore, another organ may be targeted by DMHCA and LXRs from a different cell type may be activated.

2.4 Activation of LXRs in enterocytes alters gene expression in the intestine

The liver interacts closely with the intestine, as blood travels from the intestine through the portal vein to the liver for filtering. This blood is nutrient-rich; however, it also contains many MAMPs, including LPS, that elicit inflammatory responses. The intestine acts as a barrier to reduce this exposure to proinflammatory particles; however, a failing gut barrier can contribute to chronic inflammation. This is referred to as leaky gut, which along with dysbiosis,

causes hepatic inflammation, fibrosis, and the activation of anti-apoptotic signals [18]. This insufficient barrier can promote the progression of liver disease. In one study, a high fat diet increased the intestinal permeability and LPS levels in mice [44]. Therefore, the gut and liver are highly coupled, and both play a role in NASH. In addition to being expressed in Kupffer cells, LXRα is also highly expressed in the intestine. LXRs in the intestine have been shown to reduce intestinal cholesterol absorption and stimulate reverse cholesterol transport through genes including *Abcg5* and *Abcg8*, which are more highly expressed in the intestine than the liver [30]. In addition, reduced cholesterol absorption is thought to also be due to lower levels of *Npc1l1* expression, which is responsible for the uptake of cholesterol. In an early study of LXR agonists, it was identified that DMHCA activates *Abca1* expression in the liver, macrophages, and the intestine to promote cholesterol efflux, without inducing fatty acid synthesis [29]. Therefore, the activation of LXRs in the intestine could have beneficial effects on cholesterol and lipid levels, potentially lending protection from NASH. Enterocytes are the columnar epithelial cells lining the small intestine that have been shown to respond to synthetic mimetics. Since the gene expression change was minimal in the liver after DMHCA treatment, there may be another physiological mechanism occurring elsewhere to produce beneficial histological outcome. Therefore, I hypothesize that DMHCA is acting in the intestine to stimulate LXR signaling in enterocytes to activate LXR target genes that are beneficial for liver disease.

In order to test this hypothesis, mice were placed on a control diet or a control diet with DMHCA supplemented into the food for 6 weeks. The duodenum of the intestines of these mice were harvested, and RNA was extracted, followed by whole intestine RNA-seq. Enterocytes make up a large portion of the intestinal cell population. Gene expression analysis was conducted and revealed that DMCHA had a strong effect on the intestine (Figure 14).

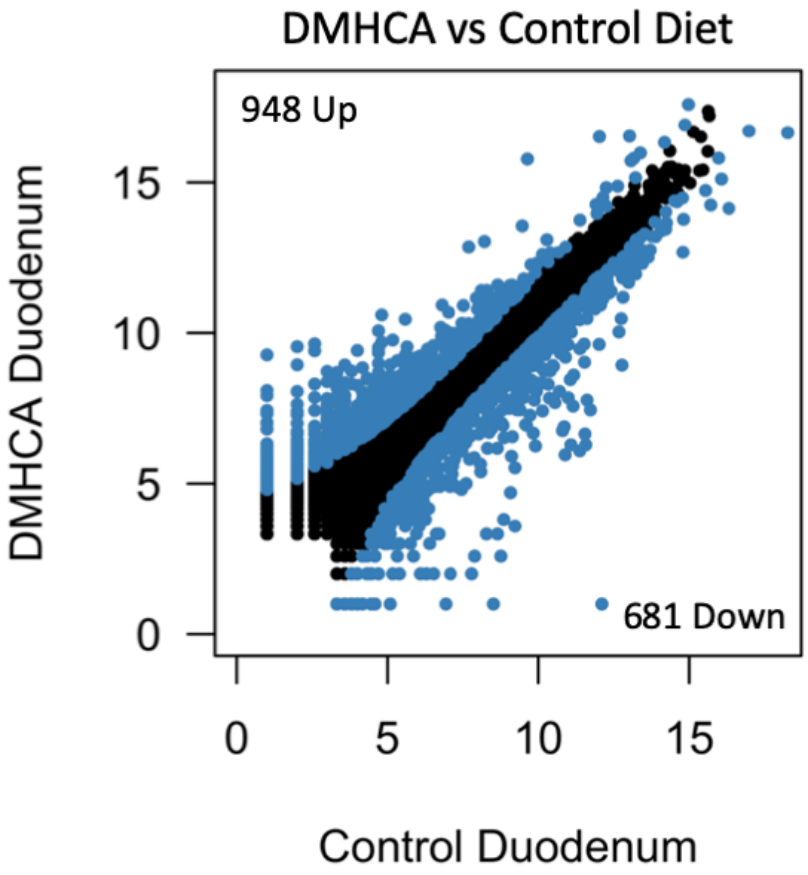

Figure 14. DMHCA treatment alters the gene expression profile of the intestine. Scatter plot of Edge R adjusted RNA-seq values from the duodenum of mice fed a control diet or a diet with DMHCA added. Each treatment group had one replicate. Blue colored points represent differentially expressed genes (FC >2 and p-value < 0.05). DMCHA has a large effect on the intestinal gene expression.

There are over 1,500 genes with altered expression levels when treated with DMHCA. This means that LXRs in the intestine are being activated by oral treatment of DMHCA. There are nearly 1,000 upregulated genes that could include genes that are beneficial during NASH, which may be the reason there was a phenotype in the previous study with DMHCA supplemented in a high-fat diet. This is very interesting because DMHCA hits the intestine before traveling to the liver, which could mean that intestinal DMCHA modulation of LXRs is all that is necessary to improve the NASH phenotype. Further investigation of intestinal gene expression changes

during NASH is necessary to confirm these findings. Therefore, a study with a large sample size with mice fed a high fat diet versus mice fed this diet with DMHCA incorporated would be beneficial to understanding these preliminary findings.

There are many differentially expressed genes in Figure 14, so investigating the expression levels of target genes of LXRs may help understand how DMHCA is altering intestinal genes. Important LXR target genes (Figure 15) include *Npc1l1*, which increases cholesterol uptake; *Abcg5* and *Abcg8*, which are responsible for cholesterol excretion; *Srebf1*, which is involved in fatty acid synthesis; and *Abca1*, which regulates cholesterol efflux.

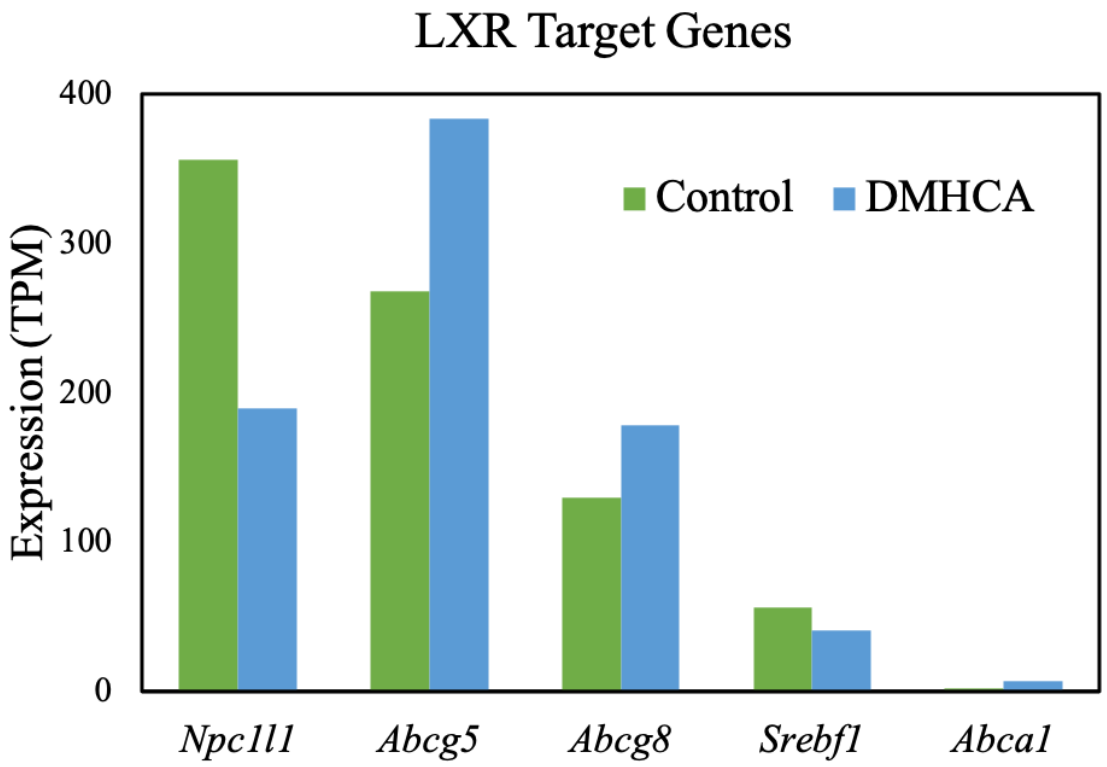

Figure 15. Gene expression level alterations of LXR target genes.
TPM expression levels of LXR target genes from intestinal RNA-seq performed on bulk duodenum from mice fed a control diet and mice fed a control diet with DMCHA incorporated. Target gene expression levels reveal potential beneficial effects on cholesterol levels.

This gene expression level analysis reveals that the cholesterol excretion genes (*Abcg5* and *Abcg8*) are increased, which means that there may be increased cholesterol expulsion leading to lower levels in the body. In addition, *Abca1* is upregulated, implying that there is more beneficial cholesterol processing. Lastly *Npc1l1* is decreased, which could mean that there is less cholesterol absorption in the intestine which may decrease cholesterol levels. Therefore, DMHCA modulated LXR activation is affecting target genes in the direction that is beneficial for cholesterol levels and fatty acid pathways. Overall, this section has revealed that DMHCA is having direct effects on the intestine, which may be interacting with the liver to modulate the NASH phenotype. An insightful follow-up study would include a larger sample size and longer time on diet, with cholesterol level tests as well as enterocyte and Kupffer cell isolations to fully understand how LXRs in these cell types are playing a role in the complex disease of NASH. However, this preliminary data shows a promising phenotype and gene expression level alterations that appear to have a positive effect on NASH.

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CHAPTER 3. METHODS

3.1 Mice

All animal procedures were approved by the University of California San Diego Institutional Animal Care and Use Committee (IACUC) in accordance with University of California San Diego research guidelines for the care and use of laboratory animals. The following mice were used in this study: C57BL/6J (The Jackson Laboratory, Stock No.000664), Clec4f-cre-tdTomato (generated by Glass Lab and transgenic core facility, University of California, San Diego), LysM-cre [45] (The Jackson Laboratory, Stock No.004781), $Nr1h3^{fl/fl}Nr1h2^{fl/fl}$ (developed by Chambon Lab), Nr1h3 knockout [22] (The Jackson Laboratory, Stock No. 013763). Mice used for these studies were between 8 and 12 weeks of age.

3.2 Mouse Diets

The NASH-model diet (Research Diets, D09100301) is composed of 40 kcal% fat from vegetable shortening (22.6% trans-fat), 20 kcal% from fructose, and 2% cholesterol by mass per 4,057 kcal. The DMHCA-NASH diet is custom manufactured to incorporate DMHCA into the NASH-model diet (Research Diets, D09100301), with a concentration of DMHCA such that each mouse received 20 mg/kg/day (Research Diets, D18112001). All food is fed *ad libitum*.

3.3 Sorting Kupffer cells

Mice were humanely euthanized by CO_2 exposure. Livers were perfused through the inferior vena cava with digestion buffer comprised of HBSS with Calcium and Magnesium (Gibco) supplemented with 0.033 mg/ml of Liberase TM (Roche), 20 μg/ml DNase I, 1 μM flavopiridol, and 20 mM HEPES. Livers were then removed, minced, and digested at 37°C with rotation. Cells were then strained, and hepatocytes were removed by low-speed centrifugation.

Next, cells were washed and resuspended in 10 ml of 28% OptiPrep (Sigma) and carfully placed beneath 3 ml of wash buffer using a Pasteur pipette to create a gradient. This was centrifuged at 1,400 X G for 25 minutes and cells at the interface were collected and red blood cells removed with RBC lysis (eBioscience). These cells were washed and suspended in PBS, followed by staining with Zombie NIR (BioLegend) and purified anti-CD16/32 (93, BioLegend) to label dead cells and block Fc receptors. Next, cells were stained for 20 minutes with desired antibodies and then washed. Cells were then sorted using a Beckman Coulter MoFlo Astrios EQ. Kupffer cells were defined as $CD146^{Low}F4/80^{Hi}CD11b^{Int}$, and dead cells and doublets were excluded.

3.4 RNA-seq library preparation

Total RNA was isolated from bulk intestine or liver tissue and purified using a Direct-zol RNA MicroPrep Kit (Zymo Research). FACS purified cells were put into lysis buffer (100mM Tris-HCl pH7.5, 500mM LiCl, 10mM EDTA pH8.0, 1% LiDS, 5mM DTT) and stored at –80°C until processing. First, mRNAs were poly A enriched by incubating with Oligo d(T) Magnetic Beads (NEB, S1419S). They were then fragmented in 2x Superscript III first-strand buffer with 10mM DTT (Thermo Fisher Scientific) by incubating at 94°C for 9 minutes. The fragmented mRNA was mixed with Random primers (Thermo Fisher Scientific), Oligo dT primer (Thermo Fisher Scientific), SUPERase-In (Ambion), dNTPs and DTT, and was heated at 50°C for one minute. After the incubation, water, DTT, Actinomycin D, Tween-20 (Sigma) and Superscript III (Thermo Fisher Scientific) were added and incubated in a PCR (polymerase chain reaction) machine. This product was then purified with RNAClean XP beads (Beckman Coulter) and eluted with nuclease-free water. The RNA/cDNA double-stranded hybrid was then added to Blue Buffer (Enzymatics), dUTP mix, RNAse H, water, DNA polymerase I (Enzymatics) and Tween-20 and incubated at 16°C for 2.5 hours. The resulting dUTP-labeled dsDNA was purified using

SpeedBeads (GE Healthcare), and then mixed with PEG8000 and 2.5M NaCl to make 13% PEG. It was then eluted with EB buffer (10 mM Tris-Cl, pH 8.5). Next, the purified dsDNA underwent end repair by blunting, A-tailing, and adapter ligation using barcoded adapters (Bioo Scientific). Libraries were PCR-amplified for 16 cycles, size selected by gel extraction, quantified using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) and sequenced on a HiSeq 4000 (Illumina).

3.5 Data Mapping

FASTQ files from RNA sequencing experiments were mapped to the mouse mm10 genome using STAR with default parameters (Dobin et al., 2013). Next, HOMER was used to create tag directories from mapped samples for further analysis (Heinz et al., 2010).

3.6 RNA-seq analysis

To quantify the gene expression raw read counts, HOMER's "analyzeRepeats" script was used with the parameters "-condenseGenes -count exons -noadj." To generate a table of TPM values, the parameters "-count exons -condenseGenes -tpm" were used. The TPM values were further converted by log2(TPM+1). DESeq2 (Love et al., 2014) analysis with the "getDifferentialExpression" HOMER command was used to identify differentially expressed genes with an FDR < 0.05 (False Discovery Rate) and FC > 2 (Fold Change). The UCSC genome browser (Kent et al., 2002) was used to visualize RNA-seq data and plots were created using R Studio.

CHAPTER 4. DISCUSSION

The functions of LXRs in Kupffer cells and their roles during the progression of NASH are largely unknown. Here, LXRs were shown to affect inflammatory genes in a knockout model, which agrees with previous studies in which LXRs were able to suppress inflammatory response genes in macrophage foam cells present in atherosclerotic lesions [24]. However, little is known about their effect on hepatic tissue, especially in Kupffer cells, which are responsible for responding to liver injury during disease. We have seen here that hepatic cell environment is altered when LXRs are lost, as there is an increase in the $Cd11b^{Int}F4/80^{Hi}Tim4^{Neg}$ cell population. Interestingly, this corelates with the result of a NASH inducing diet. Therefore, the mechanism of LXRs appears to be important for maintaining the function of Kupffer cells, and without them there is increased infiltration of monocyte-like cells. This means that LXRs may be beneficial for Kupffer cell identity and for responding to liver injury.

In addition to this increase in disease-associated monocytes when LXRs are absent during NASH, there is a major decrease in Tim4 positive resident Kupffer cells. As shown in Figure 6, not all Tim4 positive cells have LXRs floxed, revealing that the models used here to delete LXRs have some limitations. Although Lyz2 Cre is well defined and widely used throughout the research community, it recombines to eliminate LXRs in not only Kupffer cells, but other myeloid cells, which means there could be confounding factors of other cell populations losing LXRs. Therefore, a novel Clec4f Cre knockout mouse was used, which recombines with a nuclear localized tdTomato and is specific to Kupffer cells. However, the flow cytometry data showed here revealed that only a small population of cells are tdTomato positive, which are also the Tim4 negative cells. This implies that the knockout efficiency is not one hundred percent and the genomics assays are limited due to studying this small population that appear to be LXR

knockout cells. Therefore, additional samples are necessary to collect enough cells for RNA-seq. Though the specific knockout mice have some limitations, they have allowed us to look specifically at LXRs in Kupffer cells when they are absent from only this cell type rather than absent from every tissue in the body. This is an exciting advancement to understanding the role LXRs play in Kupffer cells, which will help explain the functions of LXRs in this cell type during NASH. Using Tim4 as a marker is interesting because a new population of cells was visualized, however it is difficult to fully understand the cell populations. The populations may not be completely pure due to the overlap of the Tim4 positive and negative cells, as well as the potential autofluorescence of the Tim4 negative cells. Here we knocked out both alpha and beta forms, so the low cell yields may be due to the Kupffer cells dying right after they recombine and have their LXRs deleted. This may be causing an increased turnover of Kupffer cells in NASH which means that what we appear to be testing is the effect of increased Kupffer cell turnover on NASH, rather than the effect of losing LXR signaling in Kupffer cells, since so few cells in the liver actually lose LXR signaling. Therefore, further analysis is necessary to understand why a low number of tdTomato positive cells are present, and interesting future studies could consist of knocking out only the alpha or beta forms of LXRs to yield more cells.

In order to understand the importance of LXRs during liver disease, LXRs were activated with the synthetic molecule DMCHA that is structurally similar to the natural ligand desmosterol. This compound has been shown to activate LXRs both orally and by intraperitoneal injections in mice but has not been studied in the context of NASH [29]. The studies performed here were exciting studies because there is currently no therapeutic for the treatment of NAFLD or NASH. The histological data showed a decrease in the classic phenotype of NASH, including steatosis, fibrosis and inflammation, when treated with DMCHA. This molecule is a promising

treatment because it is able to activate LXRs without turning on fatty acid synthesis genes. This is the major problem with nearly all of the synthetic LXR mimetics and the main reason that these molecules are not progressing to clinical studies. Therefore, DMCHA is a promising molecule due to its selectivity. However, the gene expression data presented here revealed only minimal alterations in the liver tissue during treatment of DMHCA to mice on a NASH inducing diet. This was an unexpected result, as a strong phenotype seen in the histology data of the liver should have alterations at the gene level. The reason for this may be explained by the liver-gut axis. There is increasing evidence that supports the importance of the intestine in sterol metabolism, which means that intestine specific activation of LXRs may be a possible drug target [29]. This would allow for the beneficial effects of increased cholesterol efflux without the potential of side effects such as turning on fatty acid synthesis in the liver. Therefore, DMCHA may be acting in the cells in the intestine, most likely in enterocytes, to activate intestinal LXRs to modulate total body cholesterol and lend protection from NASH. This may be the reason we see a healthier phenotype with DMHCA treatment. Though this study was a short-term study, we were still able to see a beneficial phenotype, which means DMCHA helps improve the development of NASH. Future directions include long term studies, such as administering a NASH diet with DMCHA incorporated for 20 to 30 weeks to uncover the effects of DMCHA during the more severe forms of NASH and the long-term progression of this disease. In addition, these histological images appear to show beneficial effects with DMCHA treatment; however, in order to confirm this finding, the quantification by a trained pathologist is necessary to reveal quantifiable alterations.

Future studies are necessary to further understand the effect that DMCHA is having in the intestine. Current data reveals that the intestinal permeability is increased in mice fed a high fat

diet, which translates to humans, as patients with NAFLD also show an increase in intestinal permeability, as well as an altered gut microbiome [44]. Preliminary findings shown here have revealed that DMCHA is modulating intestinal genes in the direction that is beneficial for cholesterol efflux and excretion. However, it is not fully known the cell type in which LXRs are being activated; therefore, the investigation of the cell type specificity of DMHCA in the intestine is an interesting follow-up study. Therefore, a study in which a NASH plus DMCHA diet is fed to a larger sample size of mice followed by the isolation of multiple cell populations and whole intestine tissue should be performed to fully understand the modulation of DMHCA in the intestine. Here, we investigated the gene expression profiles of the liver and intestine and found LXRs to be important for modulating genes involved in regulating cholesterol levels. However, in order to gain a larger picture of the effect that DMCHA is having during NASH on cholesterol and lipid levels, they should be directly measured. Therefore, future directions include collecting serum, and performing the liver enzyme tests, AST and ALT, to measure hepatic inflammation, as well as measuring serum cholesterol and triglyceride levels to assess the metabolic effects of DMHCA. In addition, to study the rate of cholesterol and total lipid excretion, cholesterol can be measured in fecal samples. If fecal cholesterol levels are increased, then DMHCA could be promoting the expulsion of cholesterol, most likely by activating *Abcg5* and *Abcg8,* as we saw in the intestinal gene expression.

In summary, the goal of this thesis was to investigate the roles of LXRs in Kupffer cells and how they function during liver disease. We have seen the results of losing LXRs, as well as activation through a synthetic mimetic. LXRs are important to modulating the inflammatory response of Kupffer cells, which reveals that they have a crucial role during NASH. There is

more to learn about LXRs and exciting future directions proposed in this discussion to further elucidate the importance of LXRs and the therapeutic advancement of DMCHA.

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