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MECHANISMS IN THE ACCELERATION OF THE REGRESSION OF LIVER INJURY IN CHRONIC LIVER FIBROSIS IN MICE

by
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ABSTRACT
OF THE THESIS OF

Noor Taan Imad for Master of Science
Major: Biochemistry

Title: Mechanisms in the Acceleration of the Regression of Liver Injury in Chronic Liver Fibrosis in Mice

Background: Sustained chronic liver injury originating from several factors including viral infection, alcohol consumption, and hepatitis may lead to hepatic fibrosis characterized by an imbalance between extracellular matrix protein synthesis and degradation. Liver fibrosis may lead to liver failure in advanced stages. Regression of liver injury occurs upon cessation of the cause of injury and thus the acceleration of the regression of liver injury might play a beneficial role in the resolution of liver function. Scar-associated macrophage with anti-inflammatory activity and a high phagocytic activity has been shown to play a role in accelerating fibrosis regression. Monoacylglycerol lipase (MAGL) is a pro-inflammatory enzyme and the rate-limiting enzyme in the degradation of monoacylglycerols, responsible for metabolizing 2-arachidonoylglycerol into arachidonic acid. Inhibition of MAGL protects against liver fibrosis. Statins and Rho-Kinase inhibitors were also described to lower inflammation in liver injury through previous studies. The inhibition of monoacylglycerol lipase, the mevalonate pathway and the Rho-Kinase pathway have shown an acceleration of the liver fibrosis resolution.

Aims: In this study, the objective was to explore various mechanisms in the acceleration of regression of liver fibrosis. Thus, we aimed to investigate the involvement of PPAR-γ-mediated pathway and the role of macrophages in the PPAR-γ-dependent acceleration of regression by MAGL inhibition, and the role of autophagy in the acceleration of the regression of liver fibrosis in mice by ROCK inhibition.

Methods: In-vivo, 12-13 weeks old C57BL/6J male mice were used and chronic liver fibrosis was induced by repetitive injections of carbon tetrachloride (0.6 ml/kg, injected intraperitoneally) twice a week for 6 weeks, and the control group was given mineral oil (vehicle). Three treatment protocols have been studied in the present thesis work. In the first, a group of mice was treated with MJN110, a potent selective MAGL inhibitor, along with GW9662, an antagonist of PPAR-γ, and acceleration of liver fibrosis was investigated after macrophage deletion using clodronate liposomes. In the second, Pioglitazone, an agonism of PPAR-γ, was used to assess the involvement of PPAR-γ-mediated pathway in the acceleration of regression of liver fibrosis. In the third treatment protocol, mice were treated with Fasudil (HA-1077), a ROCK inhibitor, and Chloroquine, an autophagy inhibitor. Fibrosis was assessed by Sirius red staining of collagen deposition in liver, and hepatic gene expression was evaluated using RT-PCR.

Results: MJN110 did not show a significant inhibition of liver fibrosis as illustrated in the quantification of collagen deposition as well as the expression of the inflammatory markers. Depletion of macrophages by clodronate liposomes, during inhibition of MAGL by MJN110 and inhibition of PPAR-ɣ pathway by GW9662, showed a significant decrease in fibrotic and inflammatory genes (TGF-β, CCL3, CCL4). Moreover, MMP13 expression significantly increased upon MAGL inhibition whereas expression of MMP13 significantly decreased after macrophage depletion upon MAGL inhibition and in the presence or absence of GW9662.
Activation of PPAR-ɣ pathway by pioglitazone showed no acceleration in the regression of liver fibrosis. A significant increase in the mRNA expression of TGF-β was observed as well as that of MMP2, whereas there was no significant change in the expression of inflammatory markers (CCL4, IL-1β, IL-6) and of MMP13.
Mice treated with Fasudil (HA-1077), and Chloroquine (CQ) showed no acceleration in the regression of liver fibrosis as evidenced by no significant change in the accumulation of collagen and in the mRNA expression of α-SMA.

Conclusion: MJN110 did not show an acceleration in the regression of liver fibrosis. Both PPAR-ɣ–dependent and independent macrophages are involved in the MJN110 effect on regression of liver fibrosis and can have both anti- and pro-fibrotic effect. MMP13 can come in part from macrophages. The involvement of PPAR-ɣ pathway and autophagy was not clearly understood in this study. Further evaluations using selective and targeted knockout models shall be used to evaluate their effect in the acceleration of the regression of liver fibrosis.

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ABBREVIATIONS

2-AG	2-arachidonoylglycerol
AA	Arachidonic Acid
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
ATG5	Autophagy-related 5
CB	Cannabinoid Receptor
CCL2/3/4/5	Chemokine C-C Motif Ligand 2/3/4/5
CCl_4	Carbon Tetrachloride
CCR2	Chemokine C-C Receptor Type 2
Col-1	Collagen-1
DAA	Direct Acting Anti-viral
DAMP	Danger Associated Molecular Patterns
ECM	Extracellular Matrix
EGF	Endothelial Growth Factor
EMT	Epithelial-to-mesenchymal Transition
EndoMT	Endothelial-to-mesenchymal Transition
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HMGB1	High Mobility Group Protein B1
HSC	Hepatic Stellate Cells
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
IL	Interleukin

IP	Intraperitoneal
IV	Intravenously
LPS	Lipopolysaccharide
LSEC	Liver Sinusoidal Endothelial Cells
LXR	Liver X Receptor
MAGL	Monoacylglycerol Lipase
MCP1	Monocyte Chemoattractant Protein-1
MG	Monoglyceride
MMP	Matrix Metalloproteinases
MMT	Mesothelial-to-mesenchymal Transition
MO	Mineral Oil
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NHS	N-hydroxysuccinimidyl
NK	Natural Killer
NKT	Natural Killer T-cell
NO	Nitric Oxide
PAMP	Pathogen Associated Molecular Patterns
PDGF	Platelet-derived Growth Factor
PPAR-α/β/ɣ	Peroxisome Proliferator Activated Receptor-alpha/beta/gamma
PRR	Pattern Recognition Receptors
ROCK	Rho-Kinase Inhibitor
ROS	Reactive Oxygen Species
SEM	Standard Error of Mean

TG	Triglyceride
TGF-β	Transforming Growth Factor-beta
TIMP	Tissue Inhibitor of Metalloproteinases
TLR	Toll-like Receptor
TNF-α	Tumor Necrosis Factor-alpha
TRAIL	TNF-α-related Apoptosis-Inducing Ligand
VEGF	Vascular Endothelial Growth Factor
α-SMA	Alpha Smooth Muscle Actin

CHAPTER I

INTRODUCTION

A. Liver

1. Anatomy and Function

In mammals, the liver is considered the largest visceral organ. It weighs about 1kg in humans.[1] The liver in humans is non-lobated whereas the liver in mice is lobated meaning that each lobe can be easily separated from the other. It consists of four lobes: right lateral, left lateral, caudate and the medial lobe where the gallbladder is embedded.[2]

The liver is composed of various kinds of cells including hepatocytes, cholangiocytes, hepatic stellate cells, Kupffer cells and the liver sinusoidal endothelial cells. The functional unit of the liver is called a lobule that takes a hexagonal shape. Each lobule is centered by a central vein, and the vertices of the hexagon form the portal triads which include portal vein, hepatic artery, and bile ducts. Cords of hepatocytes and sinusoids extend from the portal region towards the central vein. (Figure 1) [3]

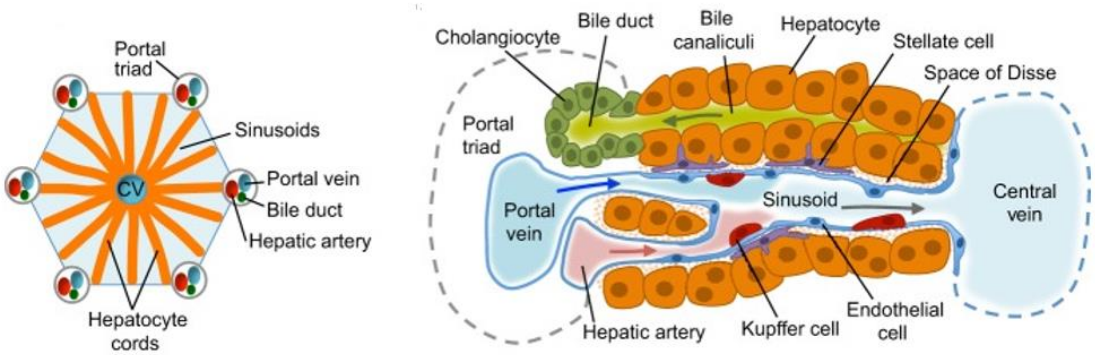

Figure 1. Liver Functional Unit [4]

Blood reaches the liver through the portal veins (75%), enriched in nutrients, and through the hepatic arteries (25%), enriched in oxygen, then leaves through the hepatic veins which makes the liver a site for exchange of molecules and elimination of toxins.[5] It is one of the largest organs in the body, and because of the variety of its cells, the liver is involved in several mechanisms essential for the survival of the body. Its function involves the metabolism of amino acids, lipids and glucose, filtration of blood, detoxification, vitamin storage, as well as having an immunological role through the production of pro-inflammatory and anti-inflammatory mediators.[6, 7] In a healthy liver, the inflammatory state is modulated where its cells can express both pro-inflammatory and anti-inflammatory mediators making the liver to be known as "tolerogenic".[8]

2. Cells of the Liver

a. Hepatocytes

Most liver cells are hepatocytes that constitute about 60-80% of the cell population.[9] They are involved in several metabolic and immunological roles such as lipids and cholesterol metabolism, production of clotting factors and albumin, antigen presentation as well as detoxification and endotoxins removal. These functions are performed through the secretion or the presence of membrane-bound proteins by the hepatocytes.[5, 10] They are also involved in the production of bile that will be modified later by cholangiocytes and then released into bile ducts to be stored in the gall bladder.[5, 11] Hepatocytes are also implicated in the innate immunity through the expression of pattern recognition receptors (PRR) and production of complement

components.[12] Cytochrome P450 enzyme is expressed by hepatocytes, that breaks down toxins or alcohol into reactive free radicals which would damage the hepatocytes themselves. Damaged hepatocytes release danger-associated molecular patterns (DAMPs) that activate other non-parenchymal cells to initiate regeneration and resolution. However, uncontrolled activation would eventually lead to liver fibrosis manifested by the accumulation of extracellular matrix (ECM) proteins.[13, 14] Hepatocytes are also involved in the production of certain alarmins like high mobility group protein B1 (HMGB1)[15] and chemokines like monocyte chemoattractant protein-1 (MCP-1, also called chemokine C-C motif ligand 2, CCL2) that recruits immune cells to the site of inflammation. Besides that, they're involved in the production of anti-inflammatory proteins to avoid excessive inflammatory reactions.[16] Anti-inflammatory reaction of hepatocytes is mediated by the activation of their liver X receptors (LXR).[8]

b. Cholangiocytes

Cholangiocytes, also known as biliary epithelial cells, are of two types: small and large that differ in their structure and function. These cells line the bile ducts.[17] They serve about 3-5% of the non-parenchymal cell population.[18] The main physiological role of cholangiocytes is modifying bile that was originally produced by hepatocytes. This modification includes several absorptive and secretory processes mainly performed by the large cholangiocytes.[11] Following liver injury, cholangiocytes proliferate and release pro-inflammatory cytokines that would recruit leukocytes in order to eliminate the pathogens. However, over-activation and excessive recruitment of these leukocytes would result in attacking cholangiocytes and

hepatocytes via an apoptotic mechanism and thus damaging the bile duct and the homeostasis of liver.[19]

c. Hepatic Stellate Cells

Hepatic stellate cells (HSCs) have been previously known as lipocytes, fat-storing cells, perisinusoidal cells, parasinusoidal cells or Ito cells.[20] They represent about 5-8% of human liver cells, are of mesenchymal origin and found in the perisinusoidal space which is also known as "space of Disse".[21, 22] In normal conditions, HSCs display a quiescent phenotype and are involved in the storage of vitamin A.[23] Upon liver injury, HSCs will be activated leading to their retinoid loss as well as their apparent expression of proliferative and fibrogenic cytokines such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β). This would promote the production of ECM in liver that hastens fibrosis.[24] They are normally star-shaped cells that would transform into myofibroblasts upon their activation.[25] It is known that HSCs are the major producing ECM proteins such as collagen (I and III mainly) and other matrix proteins like α-smooth muscle actin (α-SMA), which is also an indication of their activation.[23, 26] Studies show that HSCs also express two types of tissue inhibitors of metalloproteinases (TIMPs), TIMP 1 and 2, which restrain the action of some of matrix metalloproteinases (MMPs) and allow the progression of ECM accumulation.[27]

d. Kupffer Cells

Resident macrophages in the liver are known as Kupffer cells and occupy the liver sinusoidal lumen.[28] They serve about 20% of the non-parenchymal liver cell

population.[9] They can either originate from fetal yolk sac precursors where throughout life they would renew themselves depending on growth factors or from bone marrow-derived monocytes.[29]

Kupffer cells play an important role in maintaining liver homeostasis through its phagocytic and scavenging ability of microorganisms.[30] They also have PRR, for example toll-like receptors (TLR), that would bind to DAMPs expressed by damaged hepatocytes, and pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and flagellin leading to the production of inflammasomes, and thus promoting inflammation.[30, 31] Following liver injury, activated Kupffer cells would release different mediators and pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) [32], interleukins (IL) 1 and 6, and chemokines that would recruit other inflammatory cells.[33, 34] However, they can also play a role in the resolution of fibrosis and inflammation through phagocytosing apoptotic cells or secreting the anti-inflammatory cytokine IL-10.[34, 35]

e. Liver Sinusoidal Endothelial Cells

Liver sinusoidal endothelial cells (LSECs) are arranged as an intersection between blood circulation and hepatic cells.[36] They are the most abundant non-parenchymal cells representing about 50% of the cell population.[37] They are highly permeable due to the presence of non-diaphragmed fenestrae and lack of a basement membrane.[38] Their structure allows their communication with other liver cells, mainly hepatocytes, providing them with oxygen and nutrients from the portal circulation.[39] LSECs have additional roles including regulation of hepatic vascular blood pressure, regulation of lipid and glycoprotein homeostasis as well as immune

homeostasis, and clearance of viruses and waste products.[40] Upon liver disease, LSECs display morphological changes through a mechanism known as capillarization where fenestrae are lost, and a basal membrane is formed. They would also release pro-inflammatory mediators that would activate HSCs and hepatocytes promoting liver fibrosis and conversely affecting LSECs phenotype. [41, 42]

B. Liver Chronic Diseases

1. Liver Fibrosis

Liver fibrosis is a wound-healing response to injury or inflammation in the liver associated with the accumulation of ECM proteins (collagen, elastin, proteoglycans) and a disruption in the liver architecture.[43] It is reversible unless it reaches an advanced stage in the progression of fibrosis in which it would become known as cirrhosis. For this reason, we observe a high morbidity and mortality rate in patients suffering from liver diseases.[44]

a. Etiology

Several factors can be the reason behind the progression of liver fibrosis, for example hepatitis C or B viruses (HCV or HBV respectively), alcohol consumption, drug toxicity, acute and chronic cholestasis, metabolic and autoimmune disorders, non-alcoholic steatohepatitis (NASH) or non-alcoholic fatty liver disease (NAFLD).[45]

b. Pathogenesis of Liver Fibrosis

Liver fibrosis is demonstrated by the interplay of all liver cells regardless of the causative agent. It is initiated with the damage of hepatocytes that secrete DAMPs and

apoptotic bodies activating HSCs and Kupffer cells and leading to a cascade of inflammatory reactions (Figure 2).[46]

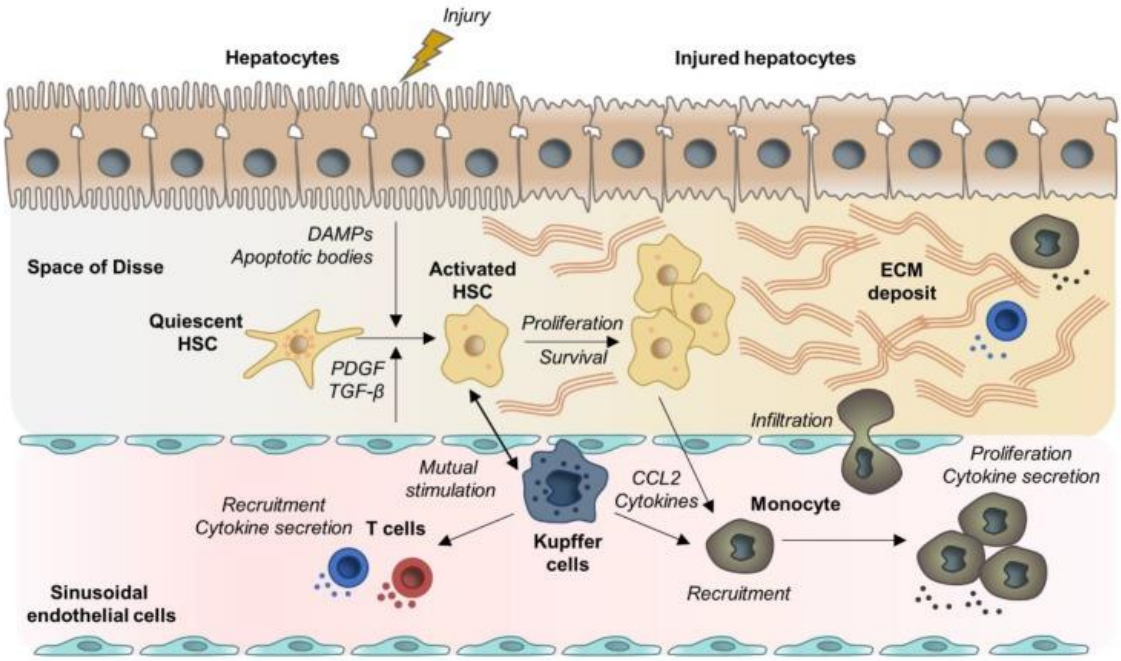

Figure 2. Mechanistic Concept of Liver Fibrosis [47]

Necrotic or apoptotic hepatocytes will also recruit leukocytes to the site of inflammation. These leukocytes would phagocytose apoptotic cells and thus amplify inflammation by releasing pro-inflammatory cytokines like TNF, IL-6 and IL-1β.[48] Both innate immune cells (neutrophils, monocytes/macrophages, natural killer NK cells) and adaptive immune cells (T and B cells) are involved in fibrosis.[49] The cytokines produced in addition to the growth factors released by the immune cells such as TGF-β, PDGF, and endothelial growth factor (EGF) would trigger the differentiation of HSCs into myofibroblasts.[46, 48] Kupffer cells would release cytokines, chemokines and reactive oxygen species (ROS) as well enhancing the differentiation process of HSCs.[50] Activated HSCs will proliferate and undergo phenotypical

changes to become involved in pro-fibrogenic responses like contractibility, chemotaxis, ECM production, α-SMA expression and leukocyte recruitment (Figure 3). [51] They also express both MMPs, that degrade ECM components, and TIMPs; however, TIMP/MMP ratio significantly increases upon full activation of HSCs causing the persistence of ECM deposition.[52]

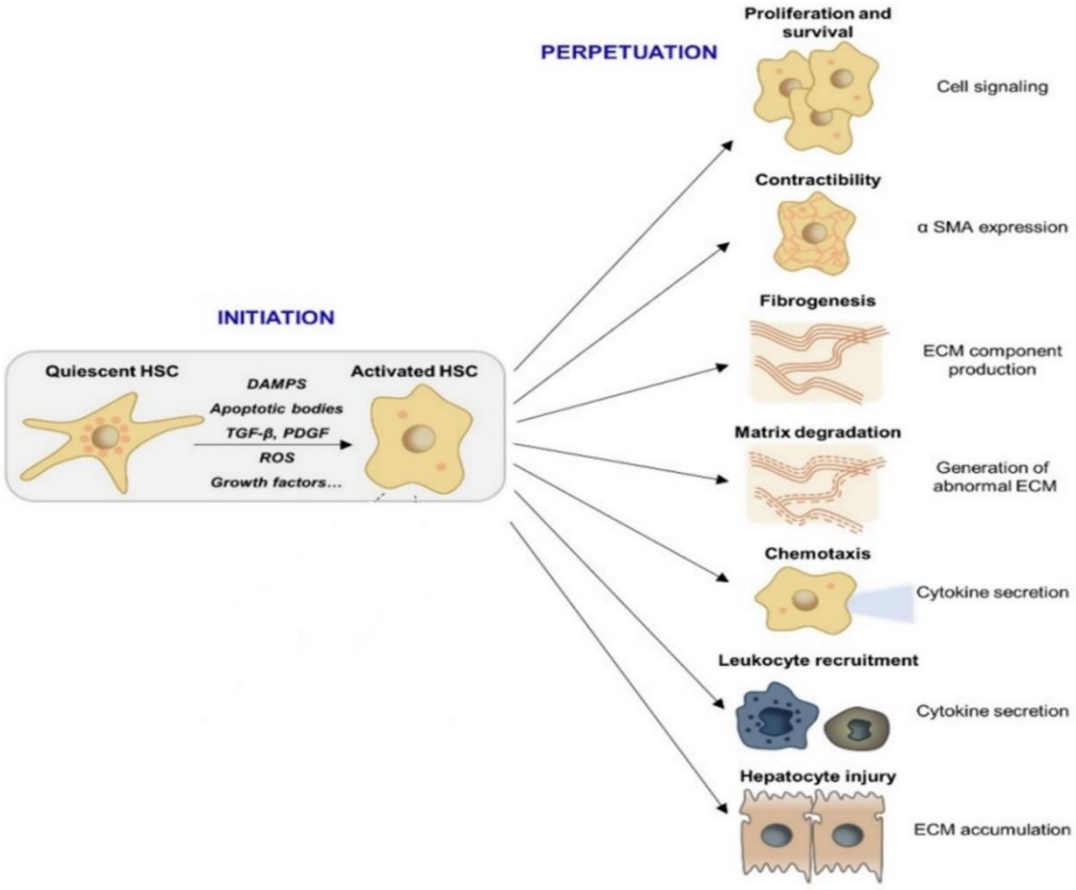

Figure 3. Pro-fibrogenic Responses upon HSCs Activation, adapted from [47]

Myofibroblasts can originate also from other cell types like tissue-resident fibroblasts, epithelial cells, endothelial cells, mesenchymal cells, fibrocytes and vascular smooth muscle cells which undergo transition known as either epithelial-to-

mesenchymal transition (EMT), endothelial-to-mesenchymal-transition (EndoMT) or mesothelial-to-mesenchymal transition (MMT) (

Figure **4**).[53]

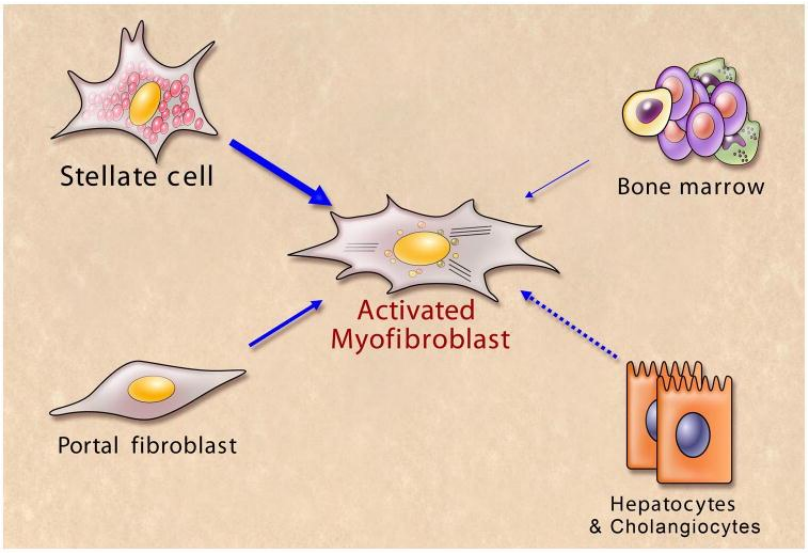

Figure 4. Origin of Activated Myofibroblasts [20]

c. Role of Macrophages, Matrix Metalloproteinases and Peroxisome Proliferator-Activated Receptors

Both macrophages and MMPs play a major role in the inflammatory and wound-healing response of the liver.

Macrophages have been divided into main types: pro-inflammatory that release inflammatory cytokines like TNF-α, IL-1, IL-6, and anti-inflammatory that release IL-10.[54] Macrophages in liver are classified as either resident Kupffer cells or monocyte-derived macrophages. Upon several studies aiming to understand the role of macrophages amid fibrosis supported with macrophage depletion (using clodronate

liposomes), it was proved that the main pro-fibrogenic macrophages are the LY6C^{hi} monocyte-derived macrophages.[48]

LY6C^{hi} monocyte-derived macrophages are recruited to the site of inflammation by the action of MCP-1/CCL2, mainly produced by the activated HSCs, and by the action of chemokine C-C motif ligands 3 and 4 (CCL3 and CCL4), also known as macrophage inflammatory proteins 1α and 1β respectively.[55, 56] Macrophages have a phagocytic activity. Moreover, they can either induce tissue damage through the secretion of ROS and nitric oxide (NO) or induce resolution through the secretion of MMPs.[56] Macrophages as well have a dual effect on HSCs by either activating them using their TGF-β and PDGF receptors or inducing their apoptosis through expression of HSCs of death ligands such as TNF-α-related apoptosis-inducing ligand (TRAIL) (Figure 5).[57]

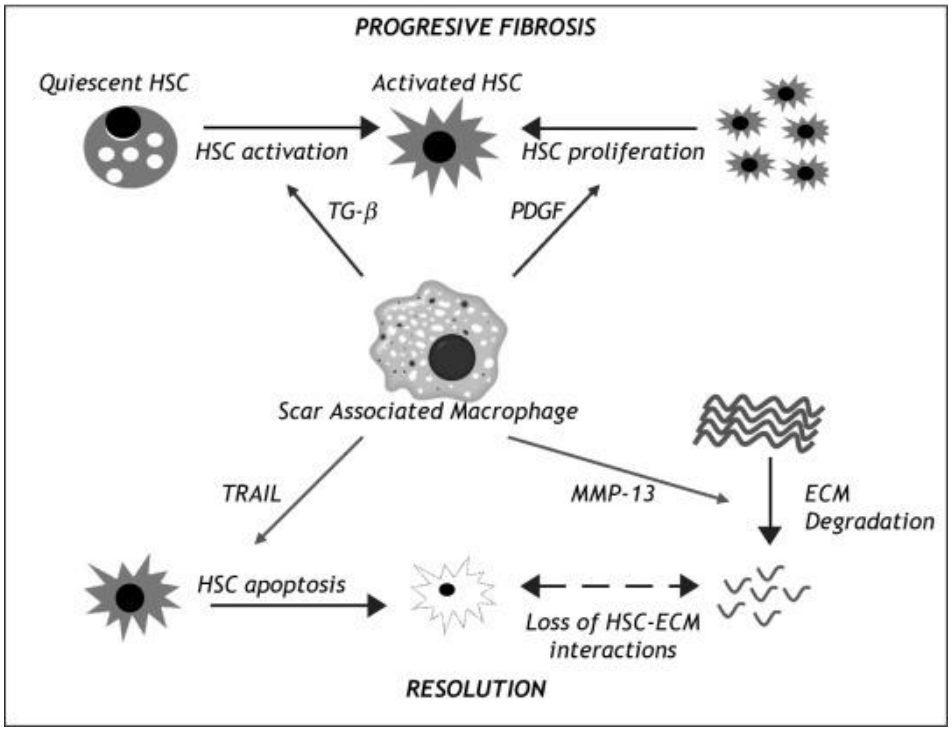

Figure 5. Dual Effect of Macrophages on HSCs, adapted from [58]

Once the stimulus causing liver injury is eradicated, macrophages can exert a significant action to re-establish the homeostasis of liver through which LY6C^{hi} monocyte-derived macrophages can convert into restorative and anti-fibrotic LY6C^{low} macrophages.[54, 59] LY6C^{low} macrophages have been seen as well as the prevalent MMP-expressing macrophages during liver fibrosis progression and resolution.[59]

MMPs are endopeptidases and classified into five groups: collagenases, gelatinases, membrane-type, stromelysins and matrilysins. They act on ECM, degrading them, and on cytokines and chemokines, regulating inflammation and immunity.[60] Some of the MMPs produced by the LY6C^{low} macrophages are MMP9, MMP12 and MMP13.[15, 61] MMPs function includes the degradation of the

accumulated ECM during progression of fibrosis. It was demonstrated that MMP12 is in charge of the degradation of elastin, the main component of elastic fibers.[62] MMP13 is a proteinase that degrades mostly collagen and gelatin. Its synthesis is regulated by TGF-β and other growth factors, and it is inhibited by the action of TIMP-1,2 and 3.[61]

In addition to what has been previously mentioned, activation of macrophages can establish homeostasis and decrease inflammation through the increased production of 2-arachidonoylglycerol (2-AG) whose role will be targeted later.[63] Furthermore, macrophages express peroxisome proliferator-activated receptor-ɣ (PPAR-ɣ) which exert anti-inflammatory effect protecting the liver from inflammation.[64]

PPARs are nuclear receptors widely spread in all tissues and are classified as PPAR-α, PPAR-δ, and PPAR-ɣ. PPAR-ɣ are involved in lipid and glucose metabolism.[65] They are also implicated in the immune response being expressed in macrophages, dendritic cells as well as T-cells and having the ability to suppress pro-inflammatory cytokines like IL-1β and TNF-α.[66] In the liver, they are expressed by hepatocytes, Kupffer cells and HSCs. PPAR-ɣ also mediate the differentiation process of macrophages favoring the restorative phenotype.[66, 67] Moreover, its activation prevents HSCs proliferation and induces their apoptosis attenuating liver fibrosis.[68]

2. Regression of Liver Fibrosis

Liver fibrosis has been addressed by many scientists to find a potential treatment. Regression of liver fibrosis is possible once the stimulus is eradicated and a sequence of anti-fibrogenic and restorative reactions dominate reversing the liver to its original state and maintaining its homeostasis. It has been clinically proven that

regression of fibrosis is possible, however reversibility of cirrhosis is still debatable and dependent on the stage of cirrhosis.[69, 70]

a. Mechanism of Regression

Regression of liver fibrosis is mediated through three main responses implemented by liver cells to acquire homeostasis of the liver: 1- Elimination of damaging agent, 2- Inactivation of HSCs or elimination of them through apoptosis, 3- Degradation of ECM.[71, 72]

Regardless of the etiology of liver fibrosis, its removal will prevent the continuous activation of hepatic cells and their intergraded inflammatory reactions.[73]

Activated HSCs either undergo apoptosis/senescence by the action of TRAIL/IL-10-12 signaling or become inactivated manifested by a decrease in its pro-fibrogenic gene expression.[49, 74] Moreover, it has been studied that PPAR-ɣ is involved in the inactivation process of myofibroblasts.[74] PPAR-ɣ expression is lost during the differentiation of HSCs, and the activation of PPAR-ɣ leads to the reversion of HSCs to their quiescent phenotype. However, these reversed HSCs have increased susceptibility to inflammatory stimuli (TGF-β and PDGF) compared to naive HSCs.[73, 75] Inactivated HSCs showed a reduction in the expression of fibrogenic genes including collagen type 1 (Col-1), α-SMA, TGF-β and TIMP-1.[76] Another study conducted by Iredale, J.P., et al confirmed that activated HSCs (α-SMA positive cells) undergo apoptosis during resolution of fibrosis, in addition to lowered levels of TIMP-1 and TIMP-2.[26] Macrophages play an essential role in inducing apoptosis of HSCs where studies have shown that their depletion in the recovery phase kept the activity and number of HSCs unchanged.[77]

During regression of fibrosis, the level of TIMPs, mainly TIMP-1, decreases aggravating the MMPs activity and thus promoting the degradation of ECM. Macrophages have the capacity to produce MMPs that aid in the degradation of the tissue scar.[72] Restorative $LY6C^{low}$ macrophages, not $LY6C^{hi}$ macrophages, are involved in the production of MMPs (MMP9-12-13).[73] Some MMPs have additional functions in the recovery phase of liver other than clearing out ECM. MMP2 can suppress the expression of Col-1[60], whereas MMP9 can induce HSC apoptosis.[78] Other mediators have been found to intermediate the ECM degradation like vascular endothelial growth factor (VEGF) by enhancing MMP2 and MMP9 expression and diminishing the production of TIMP-1 and TIMP-2 in the early stages of resolution.[79]

Macrophages have a pivotal role in the regression of fibrosis. They can produce MMPs and express survival signals to allow the regeneration of hepatocytes.[80] During resolution, Kupffer cells and infiltrating monocytes phagocytose damaged hepatocytes subsiding the release of DAMPs. This process promotes the differentiation of the pro-inflammatory into restorative macrophages.[81, 82] Campana, L., et al noticed that IL-6 triggers the phagocytic activity of macrophages.[82] The scheme below summarizes the mode of action of macrophages during regression of liver fibrosis (Figure 6).[29]

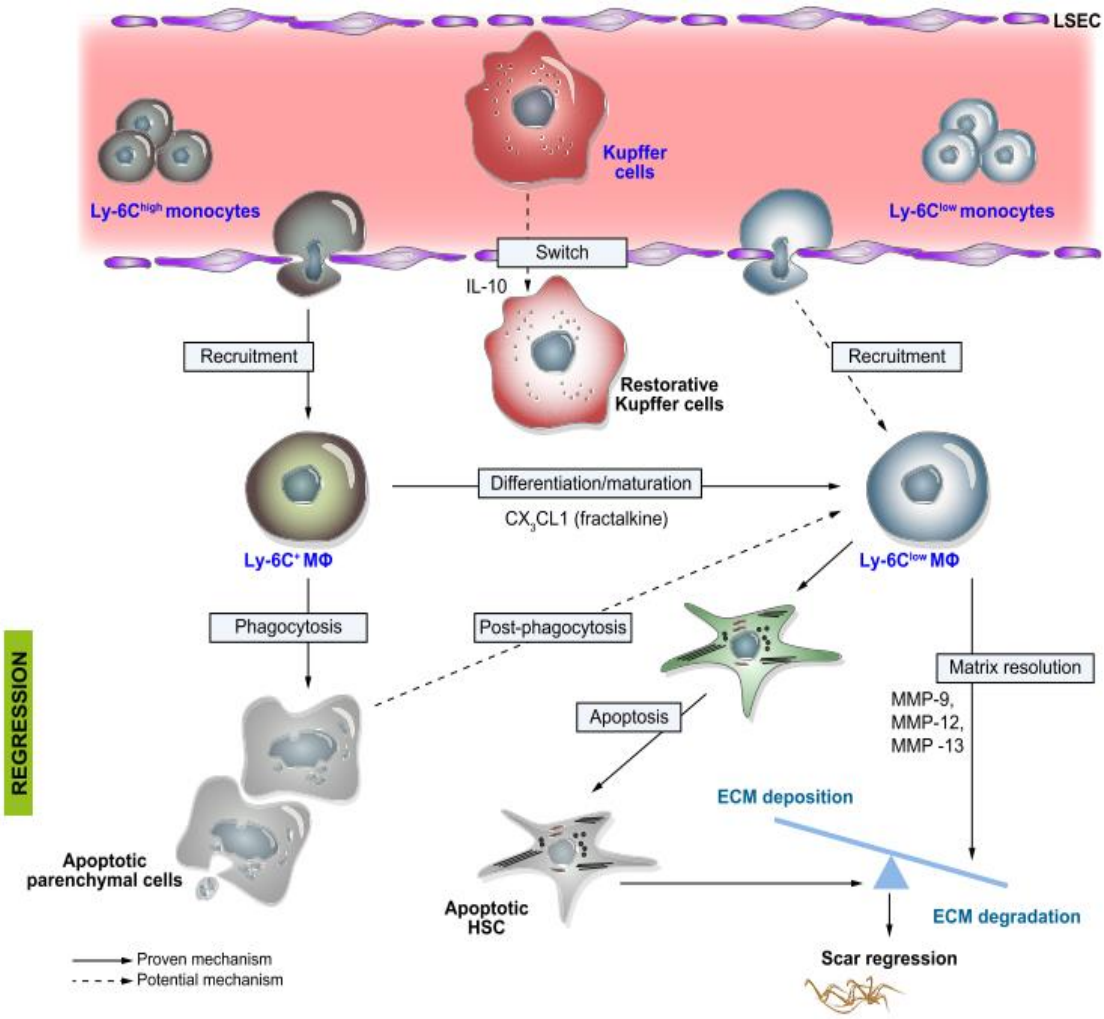

Figure 6. Macrophages During Liver Fibrosis Regression [29]

b. Inflammation in Regression

Immune cells are involved in the progression and regression of liver fibrosis and their mechanism of action relies on the chemokines and cytokines secreted by the activated cells. Each immune cell has a specific target or role, for example dendritic cells target ECM degradation though the secretion of MMP9. Others, like natural killer T-cells (NKT) and macrophages, would incite myofibroblast apoptosis.[83] Apoptosis of activated and senescent HSCs is induced by NK cells.[71] Neutrophils have been seen to exert an anti-inflammatory effect through the production of IL-1 receptor

antagonist.[84] Kupffer cells can also promote recovery through the secretion of MMP9.[85]

c. Drugs that Accelerate Regression in Mouse Models

Several drugs affecting the action of distinct effectors have been studied on mouse models of regression of liver fibrosis. For example, mNOX-E36 which is an inhibitor of MCP-1/CCL2 provided recovery of liver injury. CCL2 interacts with C-C chemokine receptor type 2 (CCR2) which is responsible for the migration of monocytes/macrophages to the site of inflammation or injury, and thus, inhibiting CCL2 decreased the infiltration of macrophages as well as their production of pro-inflammatory cytokines like TNF-α and IL-6. [86] Another study worked on inhibiting the pro-inflammatory chemokine C-C motif chemokine ligand 5 (CCL5) using the inhibitor Met-CCL5, and this showed a regression in fibrosis.[87]

The effect of resveratrol has been studied by Yu, B., et al. and they found that resveratrol stimulated the production of IL-10 by Kupffer cells facilitating resolution by activating the switch of pro-fibrotic to anti-fibrotic macrophages.[88] This study showed that resveratrol increased the number of Kupffer cells and promoted the polarization of M(LPS) into M(IL-4) associated with the increased expression of their markers inducible nitric oxide synthase (iNOS) and CD206 respectively. Moreover, resolution of fibrosis was shown through the diminished collagen deposition in treated liver as well as liver injury biomarkers like Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) [88, 89]

Inhibition of selective enzymes such as monoacylglycerol lipase showed an acceleration of the regression of liver fibrosis upon cessation of liver aggression.

MJN110, a selective and potent inhibitor of monoacylglycerol lipase (MAGL), showed an acceleration of the regression of liver inflammation and injury.[81] MJN110 belongs to the N-hydroxysuccinimidyl (NHS) carbamates family and is known as endocannabinoid hydrolase inhibitor. It acts on the active-site serine nucleophile of enzyme and inhibits it via carbamoylation. In mice, it has an IC50=2.1nM.[90] MJN110 inhibits MAGL in an irreversible manner.[91] MJN110 has lower cross-reactivity compared to other MAGL inhibitors like JZL184.[92]

d. Treatment and Therapeutical Approaches

There are several therapeutical approaches targeting liver fibrosis and clinical studies on potential drugs that would promote its regression. Liver fibrosis is diverse and thus, there are various fibrosis-associated mechanisms to target.[93] Elimination of damaging agents, for example HCV, with the use of interferon (IFN)-based or direct acting anti-viral (DAA) therapy showed regression in patients. Other therapies with anti-fibrotic effects are still under trial.[94] Another study focusing on inhibiting the inflammatory reaction has found that Cenicriviroc, an inhibitor of C-C chemokine receptor type 2 and 5 (CCR2/CCR5), provided a promising outcome in suppressing the progression of fibrosis in NASH patients. Statins have also provided beneficial effects in minimizing hepatic steatosis.[93] The effect of agonists of PPAR-ɣ, known as glitazones, on HSCs apoptosis has also been studied demonstrating positive effects in NASH patients.[77] Additional research targeting the differentiation process of macrophages into a restorative phenotype and inhibiting Kupffer cell activation is also under development.[95]

C. Monoacylglycerol Lipase

1. Structure and Function

MAGL is a serine hydrolase, made of 303 amino acids and belongs to the α/β hydrolase superfamily of enzymes. There is an 83.8% similarity between mouse MAGL and human MAGL.[96] This enzyme is present ubiquitously in all body organs such as heart, brain, liver, kidneys, and adipose tissues. It is localized in different cell compartments: plasma membrane, lipid droplets and endoplasmic reticulum.[97] MAGL is expressed in several liver cells including hepatocytes, macrophages, endothelial cells and other immune cells.[98]

MAGL is the enzyme that degrades monoglycerides (MG) into glycerol and fatty acids in a reaction known as lipid metabolism. These MG can be derived from phospholipids or triglycerides (TG).[99] It also breaks down the endocannabinoid 2-arachidonoylglycerol (2-AG), a type of MG, into arachidonic acid (AA) making MAGL a part of the endocannabinoid system.[100] Distinct enzymes can degrade 2-AG but, upon MAGL inhibition, 2-AG hydrolytic activity has been significantly diminished indicating that 2-AG degradation is mainly implemented by MAGL enzyme.[91, 101] 2-AG is an agonist to the cannabinoid receptors CB1 and CB2.[102] These receptors intervene in liver fibrogenesis and its resolution.[103] Also, it is known that arachidonic acid, the product of 2-AG hydrolysis, is involved in inflammation being the precursor of pro-inflammatory prostaglandins, for example PGE_2 and PGD_2.[63]

2. Therapeutic Applications of MAGL

Since MAGL is mediated in hydrolyzing several kinds of MG, but mainly 2-AG, which affects inflammatory responses, it serves as a therapeutic target for inflammation.[63] By inhibiting MAGL, the process will shift towards 2-AG instead of

the formation of the arachidonic acid and its pro-inflammatory prostaglandins derivatives.[104, 105]

Blocking MAGL will lead to the accumulation of 2-AG which will bind to the cannabinoid receptors, mainly CB2 receptor, increasing their signaling response and providing an anti-inflammatory reaction (Figure 7).[106] The function of the CB2 receptor was studied and it was confirmed that it mediates an anti-fibrotic impact during liver inflammation through the in-activation of HSCs, suppressing the expression of inflammatory mediators and preventing the recruitment of immune cells.[107]

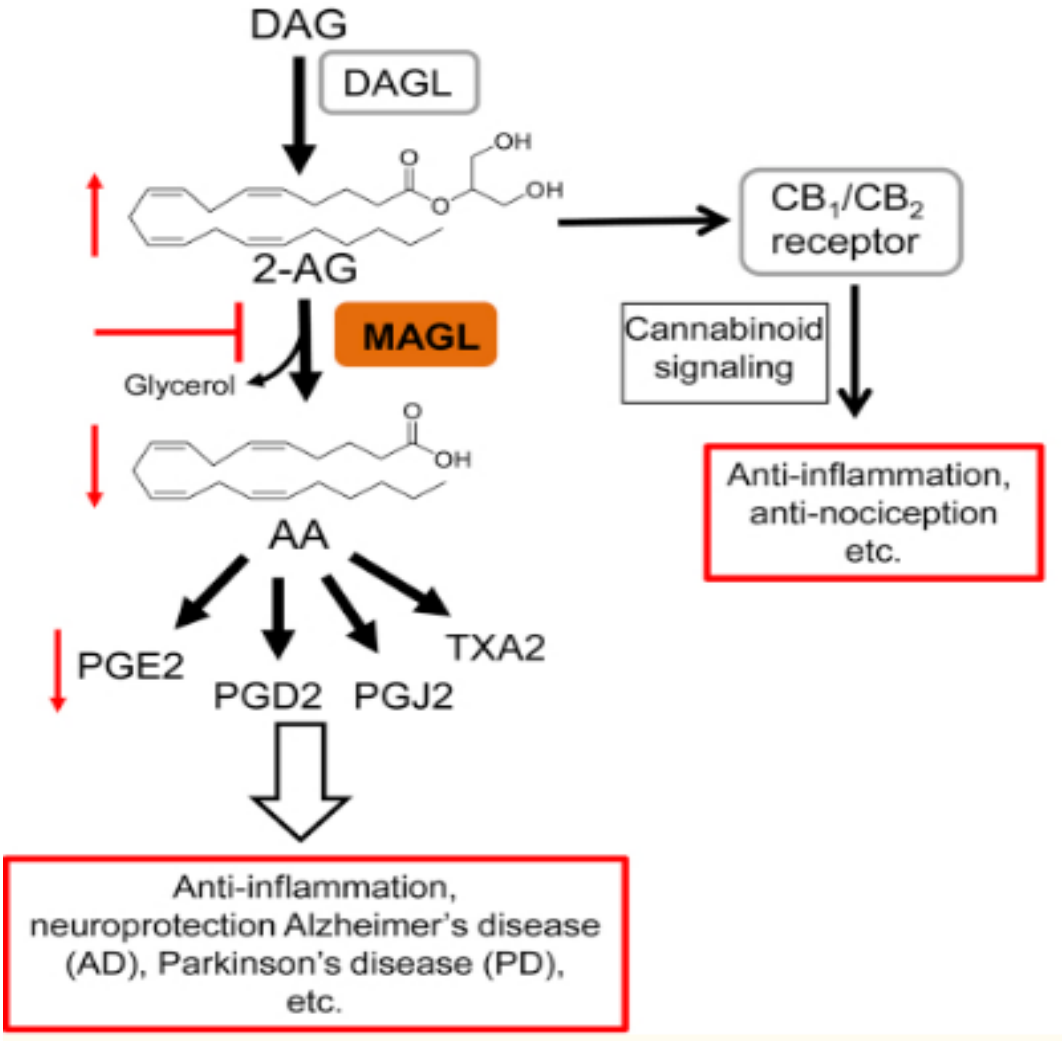

Figure 7. Inhibition of MAGL and Its Effect on the Signaling Pathway [91]

Cao, Z., et al. work also proved that MAGL inhibition increased the level of 2-AG and decreased the levels of eicosanoids (PGE_2-PGD_2) serving as a protection in CCl_4-induced acute liver injury model.[98] 2-AG can also activate PPAR-ɣ receptors that aid in decreasing inflammation.[64] Tardelli, M., et al. revealed a relation between MAGL and PPAR-ɣ with the use of pioglitazone, agonist of PPAR-ɣ, and GW9662, antagonist of PPAR-ɣ, in which MAGL inhibition allows PPAR-ɣ dependent adipogenesis leading to the prevention of hepatic steatosis.[108]

A previous study done by Habib et al. uncovered the anti-fibrogenic effect of MAGL inhibition which was determined through the decrease in the expression of pro-inflammatory cytokines and chemokines, IL1α/β and CCL2, CCL3, CCL4 respectively, in addition to a decrease in the number of F4/80 macrophages. They also proved that the anti-inflammatory property of MAGL is mediated in macrophages independently of CB2 receptors and relies on autophagy.[104]

Many inhibitors have been studied that target the active sites of MAGL among which is MJN110.[104] Most MAGL inhibitors are irreversible which desensitize the CB receptors, so it is important to synthesize reversible inhibitors to eliminate the downside effect of the irreversible ones as Jha, V., et al. are working on. They were able to discover two structurally distinct compounds that inhibit MAGL on its binding site in a reversible manner.[109] Other researchers also used a similar technique based on the structure of MAGL to discover a series of azetidine-piperazine di-amide compounds as potent and reversible MAGL inhibitors. [110]

Therefore, based on what previously has been shown and studied, MAGL inhibition serves as a protective mechanism against chronic liver inflammation and fibrosis through lowering the pro-inflammatory responses.

CHAPTER II

AIM OF THE STUDY

Previous studies in the laboratory showed that:

1- Acceleration of the regression of liver fibrosis and inflammation in a CCl_4-treated mice model by the MAGL inhibitor involved PPAR-γ signaling since the antagonist of PPAR-γ reversed the effect.

2- Fasudil (HA-1077), a Rho-kinase (ROCK) inhibitor, showed an acceleration in the regression of liver fibrosis.

3- Statins, like pitavastatin, accelerated fibrosis regression.

The objective of the present study is to explore various mechanisms in the acceleration of regression of liver injury in chronic liver fibrosis mouse model. Thus, the aim of this study is to analyze the effect of blocking some important pathways on the acceleration of regression of liver fibrosis. For this purpose, we aimed to study:

1- the PPAR-γ-mediated pathway on liver fibrosis and inflammation and the role of macrophages in the PPAR-γ-dependent acceleration of regression by MAGL inhibition

2- the role of autophagy in the acceleration of regression of liver fibrosis in mice by Fasudil (HA-1077)

CHAPTER III

MATERIALS AND METHODS

A. Animal Experiments

Experiments on mice were done using 12-13 weeks old C57BL/6J male mice. Mice were obtained from the Animal Facility of the American University of Beirut. Protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the American University of Beirut (IACUC Approval # 19-08-540).

1. CCl_4-induced Liver Fibrosis

Chronic liver injury was induced though intraperitoneal injections (I.P.) of 0.6 ml/kg carbon tetrachloride (CCl_4, Sigma-Aldrich 270652) diluted 1:10 in mineral oil (vehicle) (MO, Sigma-Aldrich M8410), twice per week for a period of 6 weeks. Control mice were injected with vehicle mineral oil (MO). Sacrifice through cervical dislocation was performed 24 and 72 hours after the last injection of CCl_4, and liver tissues were harvested at these two time-points.[111]

2. Fibrosis Regression and Mice Treatments

Mice were treated with 10 mg/kg MJN110, a specific MAGL inhibitor (Cayman Chemicals Co 17583) dissolved in PEG400-EtOH-PBS (vehicle) in a 1:1:18 ratio and was given 2 hours prior to the last CCl_4-injection and daily till sacrifice, whereas 2 mg/kg GW9662 (Cayman Chemicals Co 70785), an antagonist of PPAR-ɣ, dissolved in DMSO and drinking water (vehicle) in a 11:1 ratio was given three days

before the last CCl_4 injection and daily till sacrifice by gavage. When indicated, 2 mg/kg clodronate-liposomes (Liposoma B# C14j0319) or with an equivalent amount of control liposomes PBS (Liposoma, B# P35J0618) were administered intravenously (I.V) for macrophage depletion 12 hours after the last CCl_4-injection. Mice were divided into groups and sacrificed at the two timepoints.

Three treatment protocols were studied in the present thesis work. My involvement covered the full experimental protocol from the animal treatment to the analysis of fibrosis by histological and gene expression in regression of MJN110, GW9662 and clodronate liposomes (treatment – analysis). For the PPAR-ɣ agonist and HA-1077 studies, the animal treatment was already performed, and my role concerned the experimental procedure to detect and analyze fibrosis and the hepatic gene expression.

Fibrosis and gene expression are followed many days after the last CCl_4 injection. This allows us to examine when the resolution of fibrosis occurs and whether an acceleration of the resolution is observed.

B. Histology

1. Picrosirius Red

Collagen depositions and fibrosis were detected by Sirius red staining.[104] Upon sacrifice, one part of each of the four liver lobes were collected and fixed in 10% formaldehyde, embedded in paraffin and then 4 µm slices were cut and placed on microscopic slides. Sirius red staining was performed to detect collagen fibers deposition (Polysciences, # 24901-250). This technique was performed by Mr. Nabih Mheidly (Histology Lab, Department of Anatomy, Cell Biology and

Physiology, American University of Beirut). Eight to ten microscopic images were taken per mouse liver using the Microscope Olympus CX41 for Slide Imaging (DTS Room 308) and quantification was done using ImageJ software.

C. Gene Expression was Assessed on RNA Using Quantitative PCR with Sybergreen

1. RNA Extraction

Total RNA from the frozen liver tissues, homogenized in 1 ml QIAzol Lysis Reagent (Qiagen Lot # 55708004) using the Tissue Lyser (Qiagen II), were extracted. RNA concentrations were measured using the Nanodrop Spectrophotometer (Thermo Fisher Scientific) after resuspension in 60µl of RNase/DNase-free water. Ratio 260/280 ranged between 1.8 and 2.

2. Reverse Transcription and Real-Time PCR

2 µg of the total RNA were reverse-transcribed into cDNA using the High-Capacity Reverse Transcriptase kit (Thermo Fisher Scientific 00717406) using the RT-PCR machine (Biorad CFX384). Gene expression was determined using CAPITAL qPCR Green Mix (LRox, 4x LOT # 09231 rabbit biotech). The relative fold of gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to the housekeeping gene 18S. The selected primers for the different genes (α-SMA, TGF-β, procollagen1α, CCL3, CCL4, MMP12 and 13) were described previously. [104]

D. Data Analysis

Statistical analysis was conducted using GraphPad Prism 8, (version 8.3.0 for Windows, GraphPad Software, La Jolla, CA 92037, United States). The results are

expressed as mean ± standard error of mean (SEM). Multiple comparisons among groups were done using Mann-Whitney U test or analysis of variances (ANOVA). A *P*-value $P < 0.05$ was considered statistically significant.

CHAPTER IV

PRELIMINARY STUDIES AND RESULTS

A. Preliminary Data

Previous studies, published and unpublished, were performed in the lab to study the effect of different of drugs, including MJN110, GW9662 and HA-1077, on the progression and regression of liver fibrosis.

MJN110, a MAGL inhibitor, accelerated the regression of fibrosis and inflammation in CCl_4-induced chronic liver injury in mice. A decrease of mRNA expression of the pro-fibrotic genes (TGF-β, α-SMA), as well as of the inflammatory chemokine CCL3 were shown.[104] Moreover, increased expression of the matrix metalloproteinases (MMP12 and MMP13) which are responsible for the degradation of ECM produced was described. When the drugs MJN110 + GW9662, a PPAR-ɣ antagonist, were administered, the anti-fibrotic effect of MJN110 was obtained. The level of mRNA expression of TGF-β, α-SMA and CCL3 increased compared to that of CCl_4 + MJN110 group, and the level of mRNA expression of MMP12 and MMP13 decreased compared to that of CCl_4 + MJN110 group.

These results suggested that PPAR-ɣ participated in the MJN110-dependent effect on the acceleration of regression, and that the beneficial effect of MAGL inhibition could involve PPAR-ɣ signaling. However, the cell type involved in PPAR-ɣ signaling was not known.

Another study assessing the effect of HA-1077, a ROCK inhibitor, on liver fibrosis regression was carried out on CCl_4-induced liver injury on mice. Previous results obtained in the laboratory showed that HA-1077 accelerated the regression of

liver fibrosis with a decrease in collagen deposition in the liver and a decrease in fibrotic genes and inflammation. In parallel, HA-1077 inhibited inflammatory cytokines (IL-6) in isolated bone marrow-derived macrophages.

B. Results

1. Effect of MJN110, GW9662 and Clodronate Liposomes on the Regression of Liver Fibrosis

To assess the role of macrophages on the MJN110-dependent effect on liver regression, we first administered MJN110 (MAGL inhibitor) and/or GW9662 (PPAR-ɣ antagonist) to male C57BL/6J mice and were treated with clodronate liposomes on liver fibrosis regression. Chronic liver fibrosis was induced by CCl_4 (0.6 ml/kg) through intraperitoneal (I.P.) injections twice a week for 6 weeks. The control group was administered the same volume of the vehicle mineral oil (MO). MJN110 (10 mg/kg) or an equivalent volume of vehicle were given I.P. 2 hours before the last CCl_4 injection and daily till sacrifice. GW9662 (2 mg/kg) or an equivalent volume of vehicle were given by gavage daily starting 3 days before the last CCl_4 injection. Clodronate liposomes (2 mg/kg) or an equivalent volume of control liposomes PBS were given intravenously (I.V) 12 hours after the last CCl_4 injection (

Figure **8**).

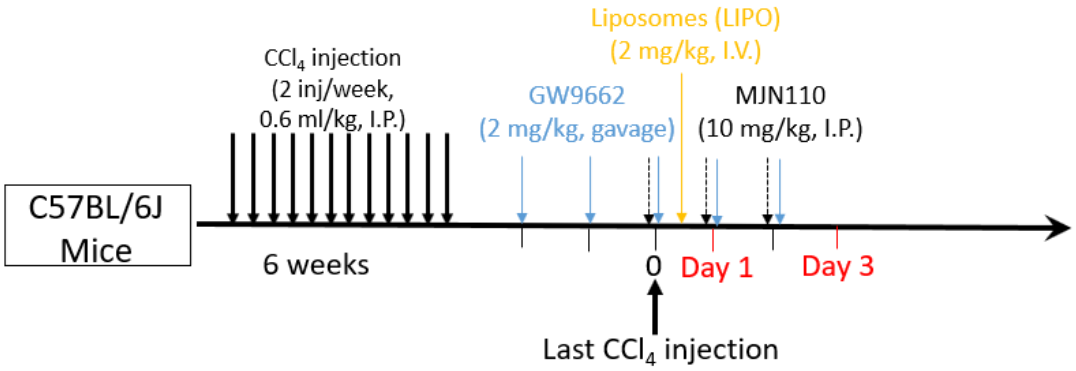

Figure 8. Schematic representation.
C57BL/6J male mice (12-13 weeks old) were treated with CCl_4 (0.6 ml/kg) twice per week for 6 weeks. MJN110 (10 mg/kg) or an equivalent volume of the vehicle (PEG400-EtOH-PBS) were administrated I.P 2 hours prior the last CCl_4 injection and daily till sacrifice, whereas GW9662 (2 mg/kg) or an equivalent volume of vehicle (DMSO) were given by gavage daily starting three days before the last CCl_4 injection. Clodronate liposomes (LIPO) or an equivalent volume of control liposomes PBS were administrated intravenously (I.V) 12 hours after the last CCl_4 injection. Mice were divided into groups to be sacrificed at two timepoints: day 1 (n=2 MO, n=7 CCl_4+vehicle, n=6 CCl_4+MJN110, n=6 CCl_4+MJN110+GW9662, n=6 CCl_4+MJN110+GW9662+LIPO, n=5 CCl_4+MJN110+LIPO) and day 3 (n=9 CCl_4+vehicle, n=7 CCl_4+MJN110, n=5 CCl_4+MJN110+GW9662, n=7 CCl_4+MJN110+GW9662+LIPO, n=5 CCl_4+MJN110+LIPO) after the last CCL_4 injection.

a. Liver Fibrosis Assessment

We first assessed the deposition of collagen fibers in liver tissues effect using Picrosirius red staining.

Figure 9 (A) shows microscopic images of liver tissues from control and treated groups at days 1 and 3. Sirius red staining showed an increase in fibrosis at days 1 and 3 of mice treated with CCl_4 alone. No clear difference was seen between the different groups.

Quantification of collagen deposition was performed using the ImageJ software, and as well showed no significant decrease in collagen deposition in any of the groups at both timepoints (Figure 9 B).

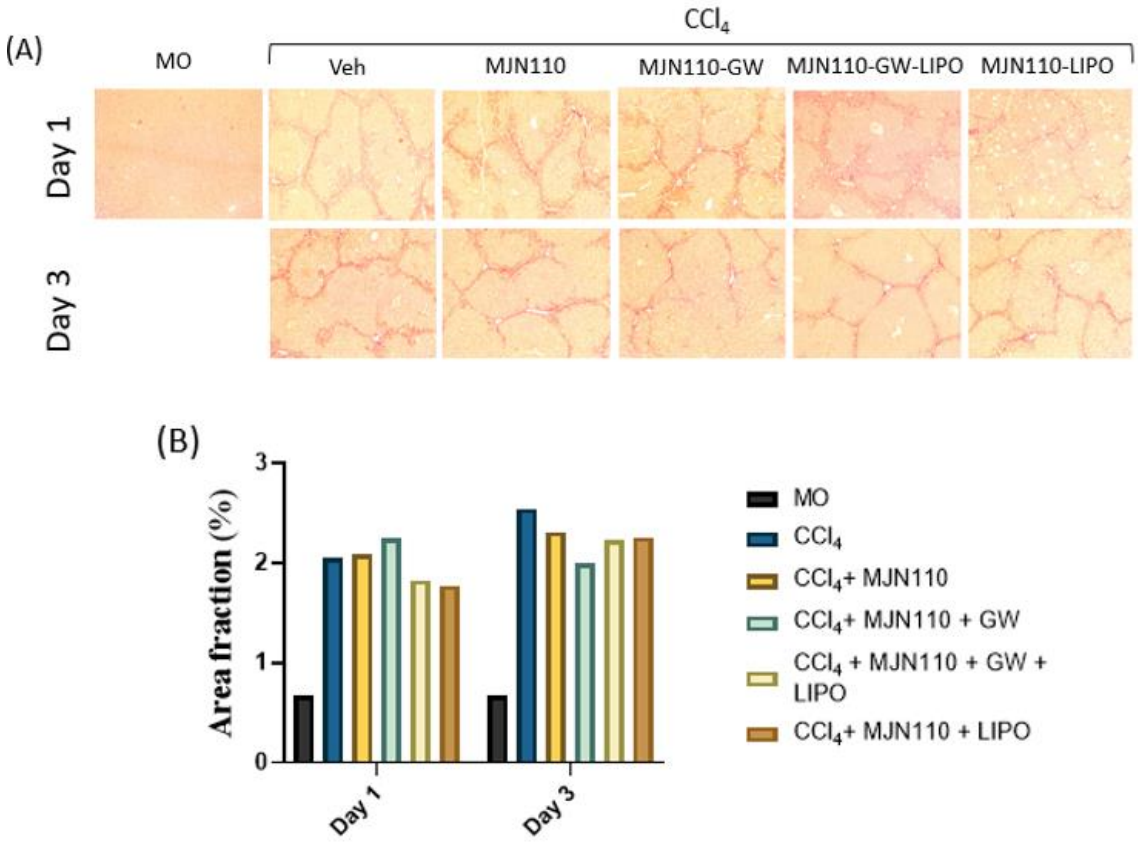

Figure 9. Effect of MJN110, GW9662 and Liposomes on Sirius red staining of liver section.
(A) Representative images of collagen deposition stained with Picrosirius Red stain in control mineral oil (MO) and treated mice at days 1 and 3 as described in (A). Magnification (40X).
(B) Quantification the area of fibrosis in liver. Sirius red staining was quantified using ImageJ and results are expressed as percentage of total area. 8-10 images of the 2-4 lobes were quantified for each mouse. Data are expressed as mean ± SEM (2way ANOVA).

In summary, in this experiment, MJN110 did not show a significant inhibition in liver fibrosis and no additional modification was clear.

b. Gene Expression of Hepatic Fibrotic and Inflammatory Genes in Response to MJN110, GW9662 and Clodronate Liposomes in a CCl_4 Regression of Fibrosis

Analysis of inflammatory markers, fibrotic markers and MMPs were therefore assessed using RT-PCR to assess the MJN110 modification and to test the effect of PPAR-ɣ antagonist and/or clodronate liposomes.

i. Effect on Fibrotic Gene

Figure 10 shows an increase in the expression of the pro-fibrotic gene TGF-β in CCl_4-treated mice compared to the control MO group. Although the decrease of TGF-β by MNJ110 was not statistically significant, a significant decrease in CCl_4 + MJN110 + Lipo group was described compared to CCl_4 + MJN110. This effect is difficult to explain and suggest that the clodronate liposome dependent macrophages or phagocytic cells are important and play a role in fibrosis, and their elimination is anti-fibrotic for TGF-β. Moreover, the reversal tendency effect by GW9662 on MJN110 (CCl_4+MJN110+GW9662) was blocked by the treatment with clodronate liposomes.

This suggest that the group of macrophages involved in the regression effect of MJN110 has both anti- and pro-fibrotic effect since the level of expression of the gene is as low as mineral oil.

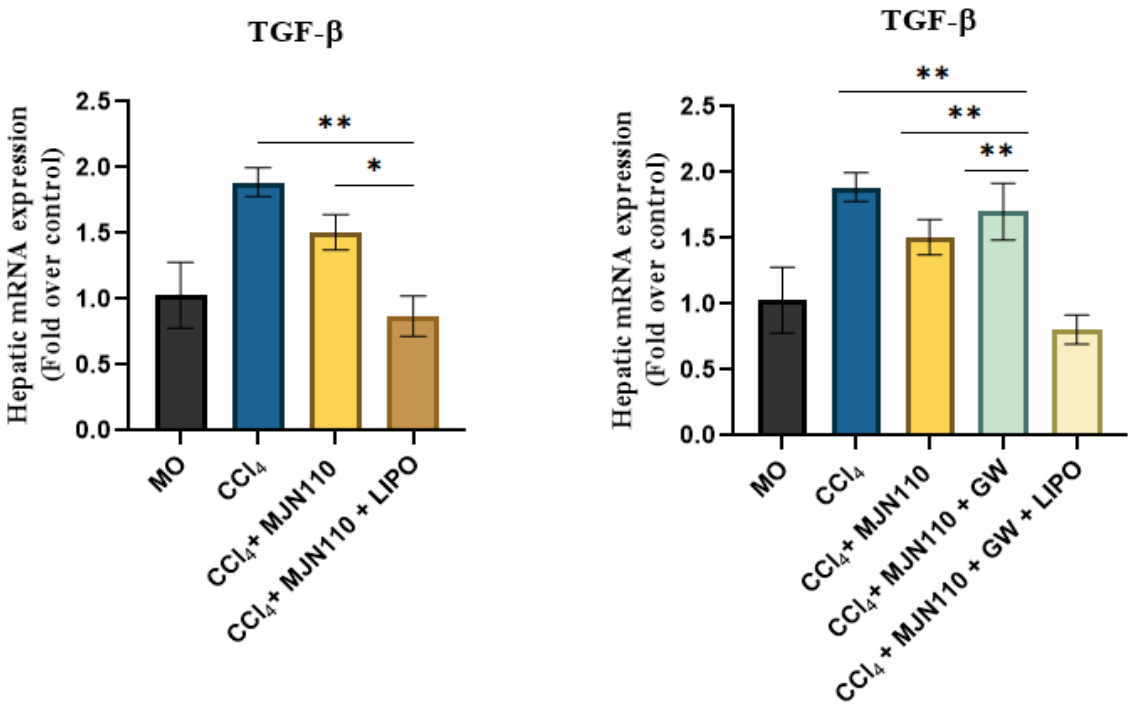

Figure 10. Expression of TGF-β in control and treated mice.
Data are expressed as mean ± SEM; * *P*-value ≤ 0.05, ** *P*-value ≤ 0.01. (Mann-Whitney U test)

ii. Effect on Inflammation

Figure 11 shows that CCL3 and CCL4 gene expression in liver was increased in CCl_4-treated group. MNJ110 alone did not show a statistically modification of the gene expression compared to CCl_4. Significant decrease in the inflammatory markers CCL3 and CCL4 when mice were treated with MJN110 and Lipo simultaneously compared to CCl_4-treated mice, as well as when the drugs MJN110, GW9662 and Lipo were administered altogether compared to the other treated groups. Clodronate alone or with GW9662 limited the level of gene expression of inflammatory genes CCL4 and CCL3 to go back to a level lower than that of CCl_4-treated group.

Results show, similarly to Sirius red results, that MNJ110 did not modify the inflammatory markers and that clodronate decreased the pro-inflammatory macrophages resulting in the decrease of inflammation in the liver.

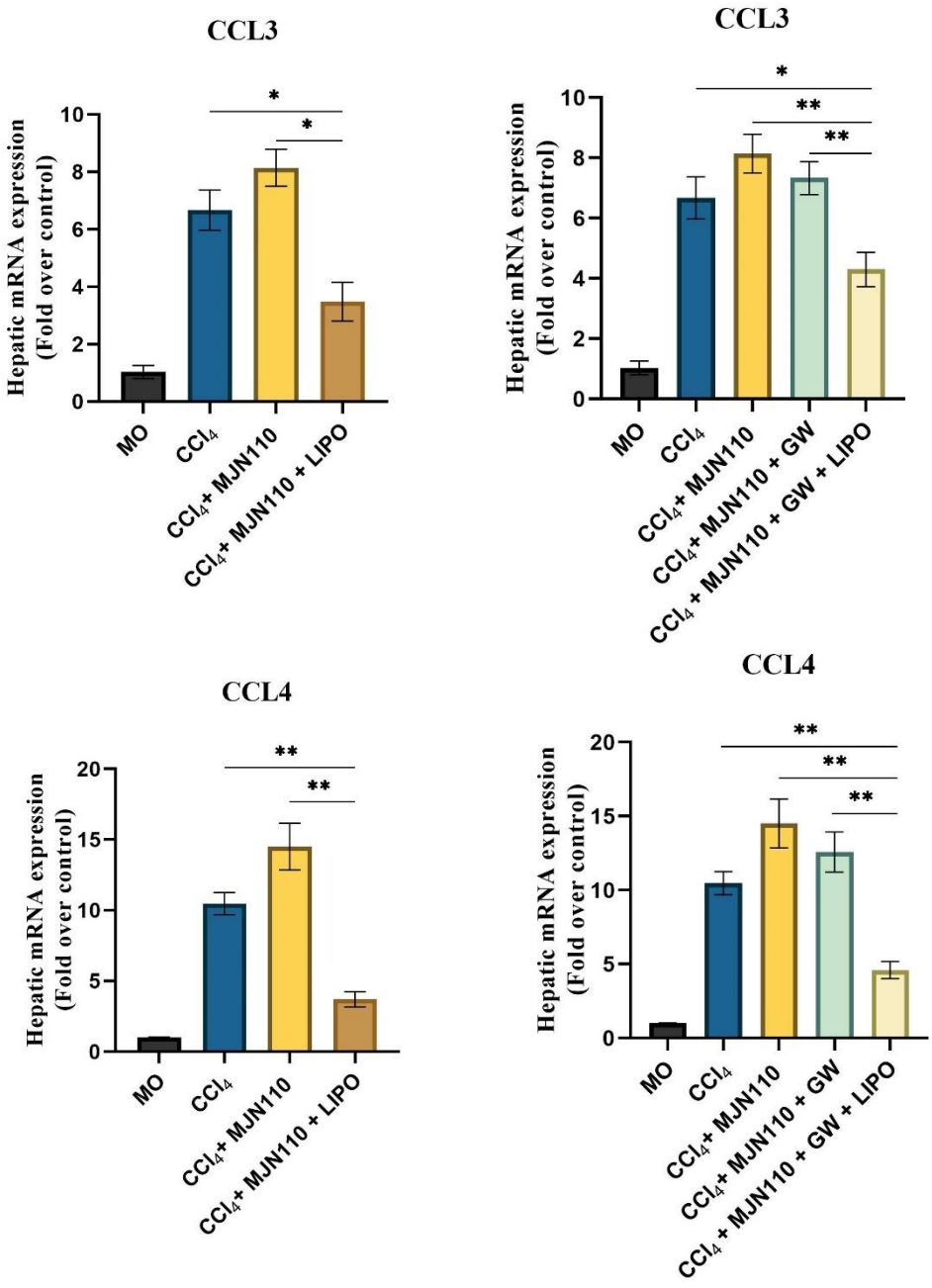

Figure 11. Expression of CCL3 and CCL4 in control and treated mice.
Data are expressed as mean ± SEM; * *P*-value ≤ 0.05, ** *P*-value ≤ 0.01. (Mann-Whitney U test)

iii. Effect on MMP13 Expression

Since MMP13 played an important role in the regression of liver fibrosis, we assessed its expression in the different mice treated groups. The gene expression of MMP13 showed a significant increase in CCl_4 + MJN110 compared to CCl_4-treated group. The gene expression of MMP13 significantly decreased in CCl_4 + MJN110 + Lipo group suggesting an important role of restorative macrophages in the effect observed by MJN110 on MMP13. Similarly, GW9662 reversed the anti-fibrotic effects of MJN110. Moreover, the expression of MMP13 decreased significantly in CCl_4 + MJN110 + GW + Lipo group compared to the other treated groups, to a level similar to CCl_4 + MJN110+ Lipo, suggesting both PPAR-ɣ–dependent and independent macrophages play a role in the MJN110 effects on MMP13 (Figure 12).

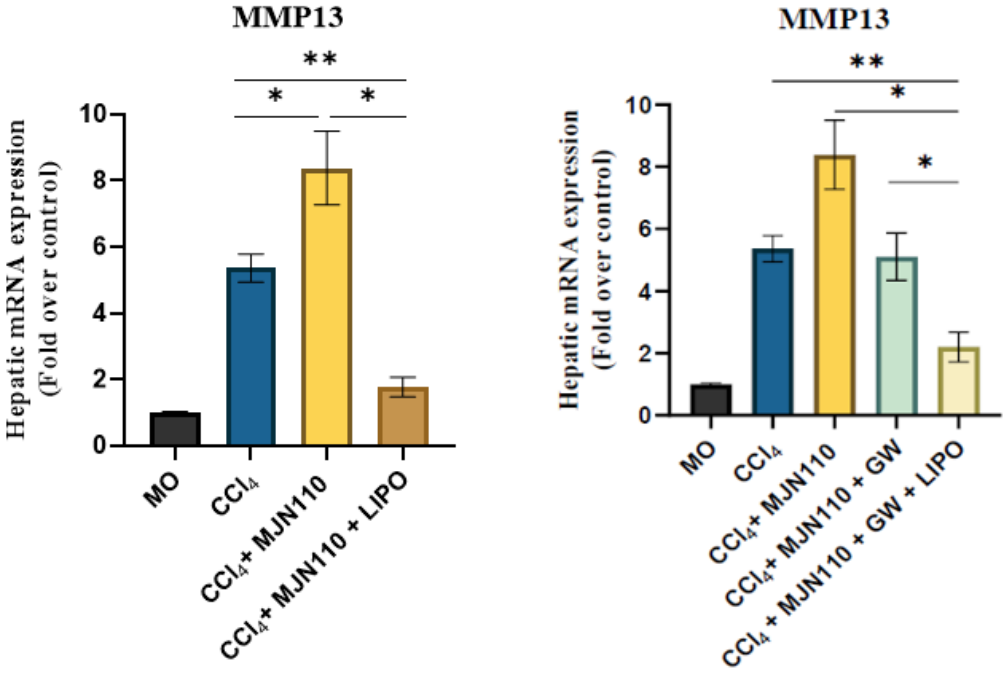

Figure 12. Expression of MMP13 in control and treated mice.
Data are expressed as mean ± SEM; * *P*-value ≤ 0.05, ** *P*-value ≤ 0.01. (Mann-Whitney U test)

2. *Effect of Pioglitazone on the Regression of Fibrosis in C57BL/6J Mice Treated with CCl_4*

In parallel, we analyzed the effect of PPAR-ɣ activation on the regression of fibrosis in a CCl_4-treated mice.

a. Schematic Representation

We studied the effect Pioglitazone (Pio), a PPAR-ɣ agonist, on liver fibrosis regression. C57BL/6J male mice were treated with CCl_4 (0.6 ml/kg) intraperitoneally (I.P.) twice a week for 6 weeks. The control group was given the same volume of the vehicle mineral oil (MO). Pio (10 mg/kg) or an equivalent volume of vehicle were given I.P. daily starting the day of the last CCl_4 injection. Mice were sacrificed at two timepoints and liver was collected for analysis (

Figure 13).

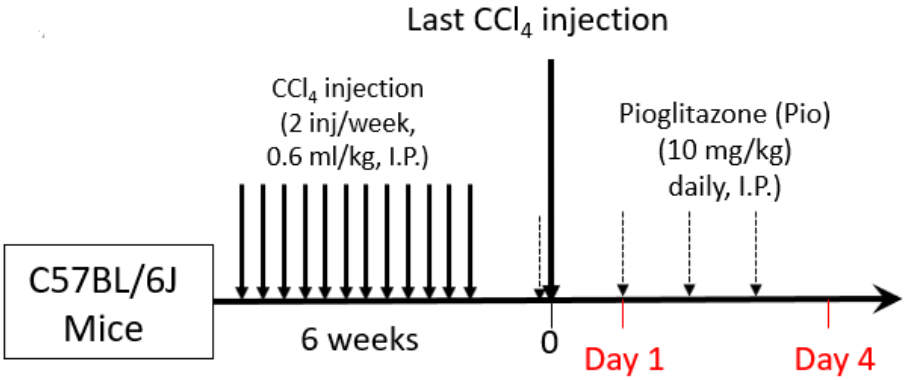

Figure 13. Schematic representation
C57BL/6J male mice (12-13 weeks old) were treated with CCl_4 (0.6 ml/kg) twice per week for 6 weeks. Pioglitazone (10 mg/kg) or an equivalent volume of the vehicle (DMSO) were administrated intraperitoneally (I.P) daily for four consecutive days starting the day of the last CCl_4 injection. Mice were divided into groups to be sacrificed at two timepoints: day 1 (n=3 MO, n=9 CCl_4+vehicle, n=6 CCl_4+Pio) and day 2 (n= 9 CCl_4+vehicle, n=9 CCl_4+Pio) after the last CCl_4 injection.

b. Effect of Pioglitazone on Blood Glucose Level

To check the effectiveness of Pio, serum blood glucose level was assessed using Accu-Chek strip at day 1. A significant decrease in glycemia was observed in CCl_4 + Pio group compared to CCl_4-treated group (Figure 14).

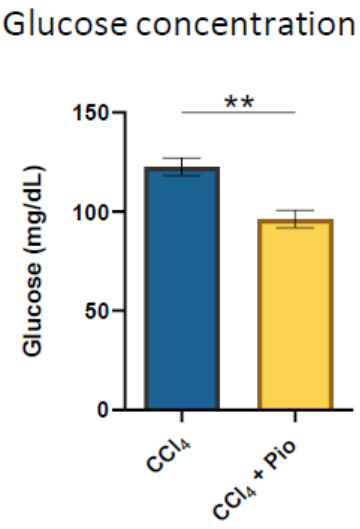

Figure 14. Blood Glucose
A drop of tail blood was collected, and glucose assayed using an Accu-Chek strip. Data are expressed as ± SEM (n=7 CCl_4, n=9 CCl_4 + Pio); ** *P*-value ≤ 0.05 is considered significant. (Mann-Whitney U test).

c. Liver Fibrosis Assessment

Collagen deposition is a marker of fibrosis and thus it was assessed using Picrosirius red staining to see whether Pio has an anti-fibrotic effect.

Figure 15 (A) shows microscopic images of liver tissues from control and treated groups at days 1 and 4. They did not show any significant change of staining among the different groups. Quantification of collagen deposition was performed using the ImageJ software, and as well showed no significant decrease in collagen deposition

in any of the groups at both timepoints. (Figure 15 B). In this experiment, Sirius red staining quantification showed a higher level of fibrosis at day 3 compared to day 1.

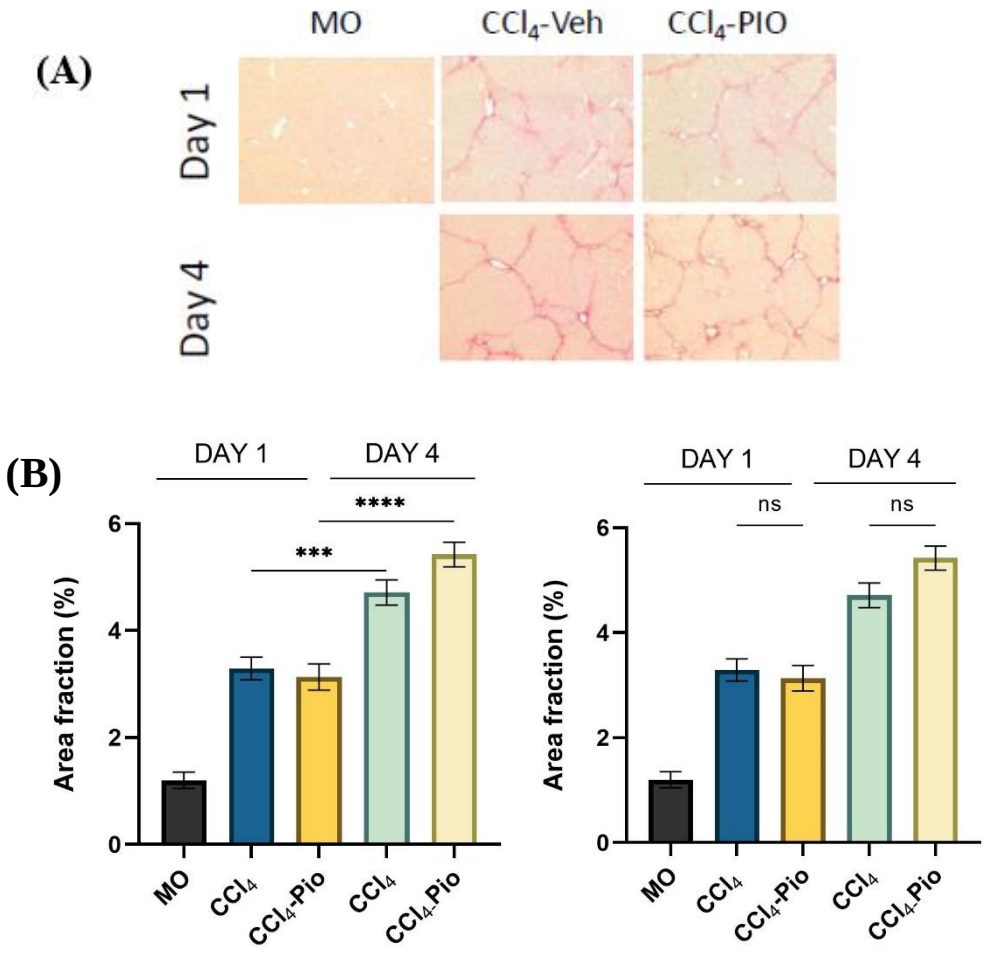

Figure 15. Pioglitazone effect on Sirius red staining of liver section.
(A) Representative images of collagen fibers stained with Picrosirius red stain in control mineral oil (MO) and treated mice at days 1 and 4 after the last CCl_4 injection in the absence or presence of pioglitazone (magnification 40X).
(B) Sirius red staining was quantified using ImageJ software and results are expressed as percentage of total area. 8-10 images of the 2-4 lobes were quantified for each mouse. Data are expressed as mean ± SEM; ns not significant, *** *P*-value ≤ 0.001, **** *P*-value ≤ 0.0001. (One Way ANOVA)

d. Gene Expression of Hepatic Fibrotic and Inflammatory Genes in Response to Pioglitazone in a CCl_4 Regression of Fibrosis Model.

Expression of hepatic genes was performed by RT-PCR. Data are shown as minimum to mean ± SEM (n=3 MO, n=6-9 CCl_4, n=7-9 CCl_4-Pio).

i. Effect of Pioglitazone on Fibrotic genes

The expression of the pro-fibrotic genes pro-collagen 1α and TGF-β significantly increased in CCl_4-treated group compared to MO. No significant decrease in mRNA level of pro-collagen 1α in CCl_4 + Pio compared to CCl_4-treated group whereas the expression of TGF-β increased significantly (Figure 16).

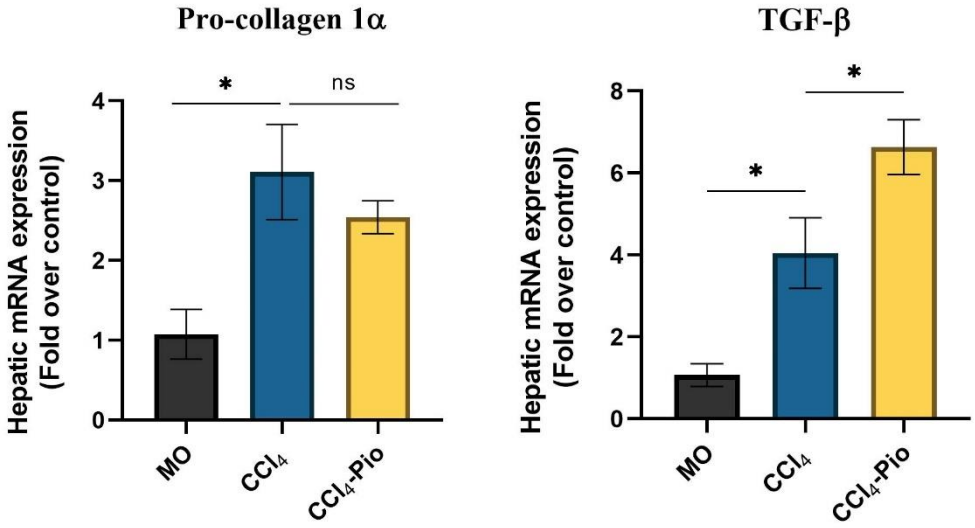

Figure 16. Expression of pro-collagen 1α and TGF-β in control and CCl_4 treated mice in the absence or presence of pioglitazone.
Data are expressed as mean ± SEM; * *P*-value ≤ 0.05 is considered significant, ns not significant. (Mann-Whitney U test)

ii. Effect of Pioglitazone on MMPs

MMP2 and MMP13 showed a significant increase in their expression in CCl_4-treated group compared to the control (MO) group. The mRNA expression of MMP2 significantly increased in CCl_4 + Pio group compared to CCl_4-treated group. Whereas there is so significant increase in mRNA expression of MMP13 (Figure 17).

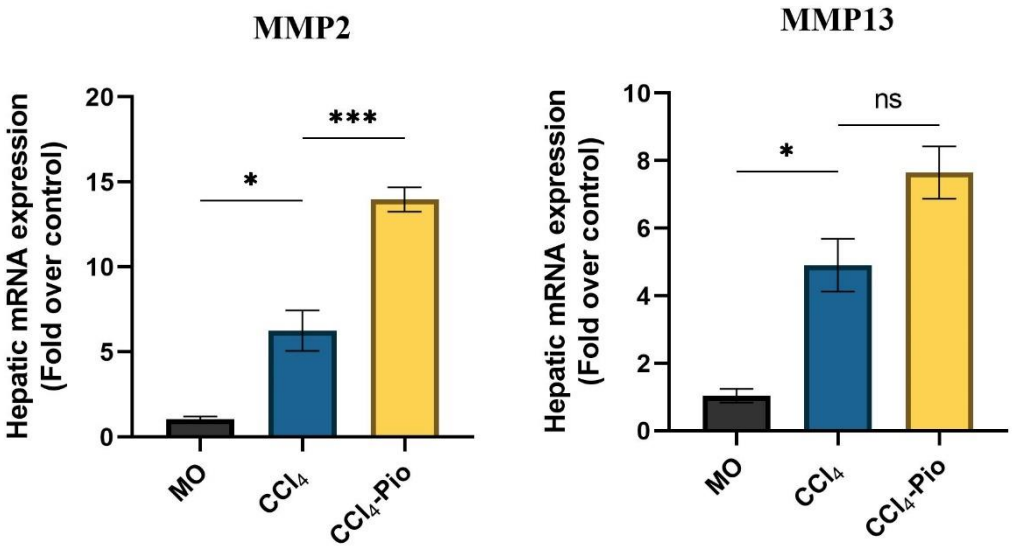

Figure 17. MMP2 and MMP13 expression in control and CCl_4 treated mice in the absence or presence of pioglitazone.
Data are expressed as mean ± SEM; * *P*-value ≤ 0.05 is considered significant, *** *P*-value ≤ 0.001. (Mann-Whitney U test)

iii. Effect of Pioglitazone on Inflammation

The expression of inflammatory mediators, CCL4, IL-1β and IL-6, were assessed. All three genes show a significant increase in CCl_4-treated group compared to the control. No significant change of mRNA expression of CCL4, IL-1β or IL-6 in CCl_4 + Pio treated group compared to the CCl_4-treated (

Figure 18).

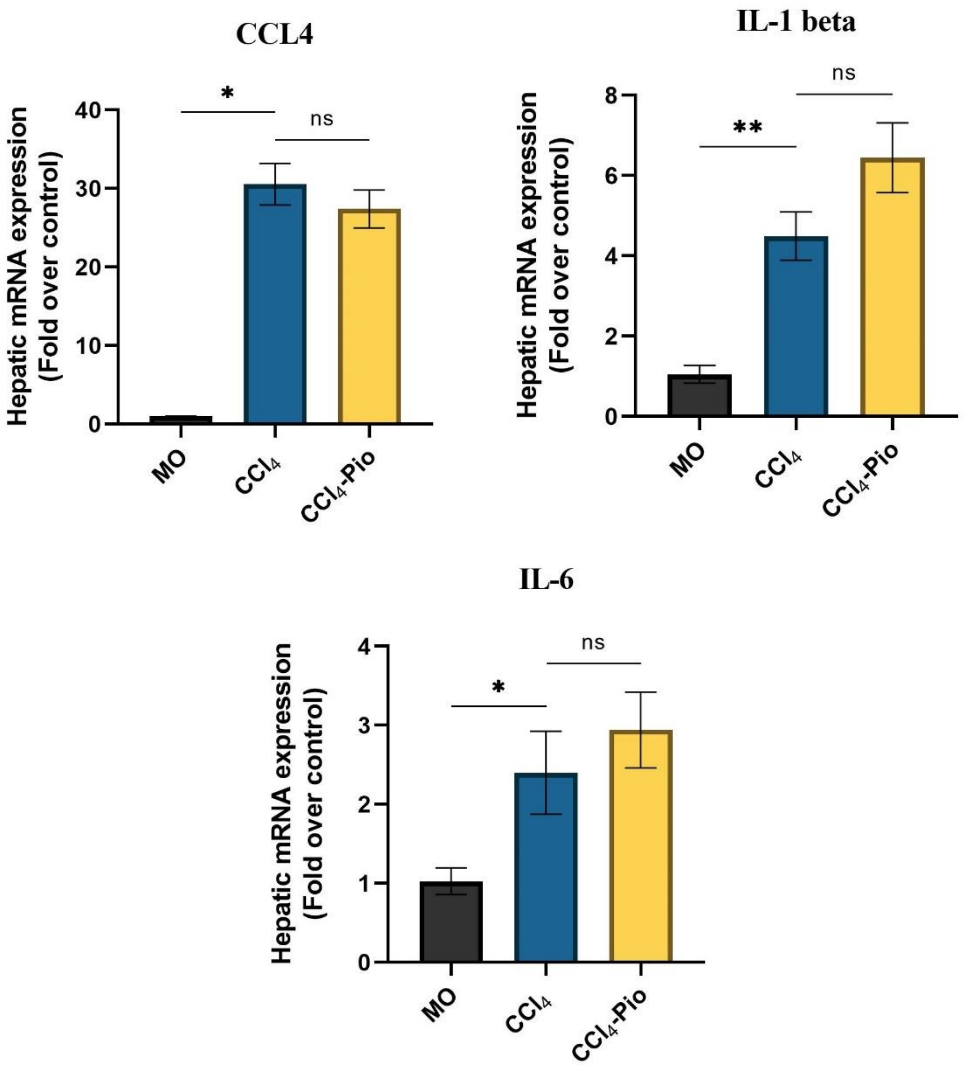

Figure 18. Inflammatory genes expression including CCL4, IL-1 beta and IL-6. Data are expressed as mean ± SEM; ns not significant, * *P*-value ≤ 0.05, ** *P*-value ≤ 0.01 (Mann-Whitney U test).

3. *Effect of HA-1077 and Chloroquine on the Regression of Fibrosis in C57BL/6J Mice Treated with CCl_4*

a. Schematic Representation

We investigated the effect of HA-1077, a ROCK inhibitor, and chloroquine (CQ) on the regression of liver fibrosis. C57BL/6J male mice were treated with CCl_4 (0.6 ml/kg) intraperitoneally (I.P.) twice a week for 6.5 weeks. The control group was given the same volume of the vehicle mineral oil (MO). HA-1077 (10 mg/kg) or an equivalent volume of vehicle were given I.P. daily starting the day of the last CCl_4 injection. CQ (60 mg/kg) or equivalent volume of vehicle were given I.P. 24 hours before the last CCl_4 injection and daily until sacrifice (Figure 19).

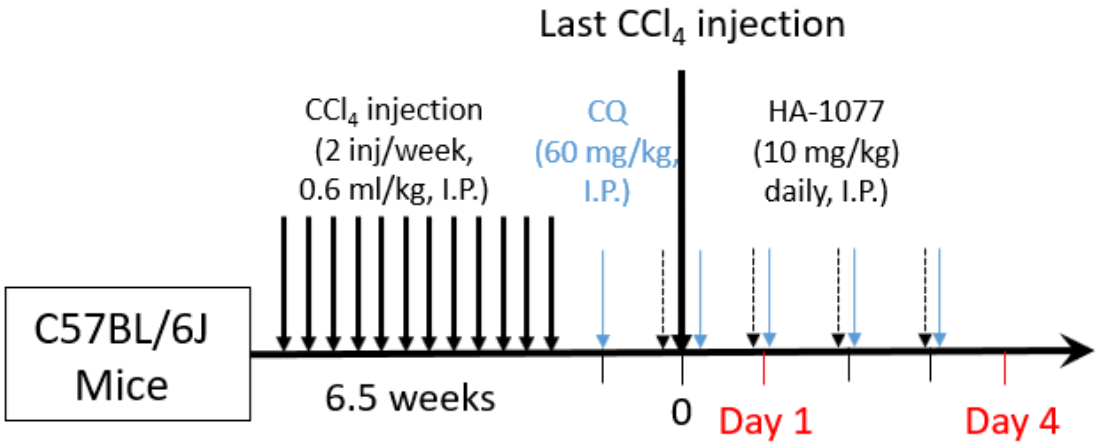

Figure 19. Schematic representation
C57BL/6J male mice (12-13 weeks old) were treated with CCl_4 (0.6 ml/kg) twice per week for 6.5 weeks. HA-1077 (10 mg/kg) or an equivalent volume of the vehicle (saline) were administrated I.P daily starting last CCl_4 injection, whereas CQ (60 mg/kg) or an equivalent volume of vehicle were given I.P one day before the last CCl_4 injection and daily until sacrifice. Mice were divided into groups to be sacrificed at two timepoints: day 1 (n=3 MO, n=8 CCl_4+vehicle, n=8 CCl_4+HA-1077, n=8 CCl_4+HA-1077+CQ) and day 4 (n=7 CCl_4+vehicle, n=10 CCl_4+HA-1077, n=8 CCl_4+CQ, n=8 CCl_4+HA-1077+CQ) after the last CCl_4 injection.

b. Liver Fibrosis Assessment

To check whether HA-1077 and CQ have an anti-fibrotic effect, Sirius red staining was performed to assess the deposition of collagen fibers in mice liver tissues.

Figure 20 (A) shows microscopic images of liver tissues from control and treated groups at days 1 and 4. They did not show any significant change of staining among the different groups. Quantification of collagen deposition was performed using the ImageJ software, and as well showed no significant change in collagen deposition in any of the groups at both timepoints (Figure 20 B).

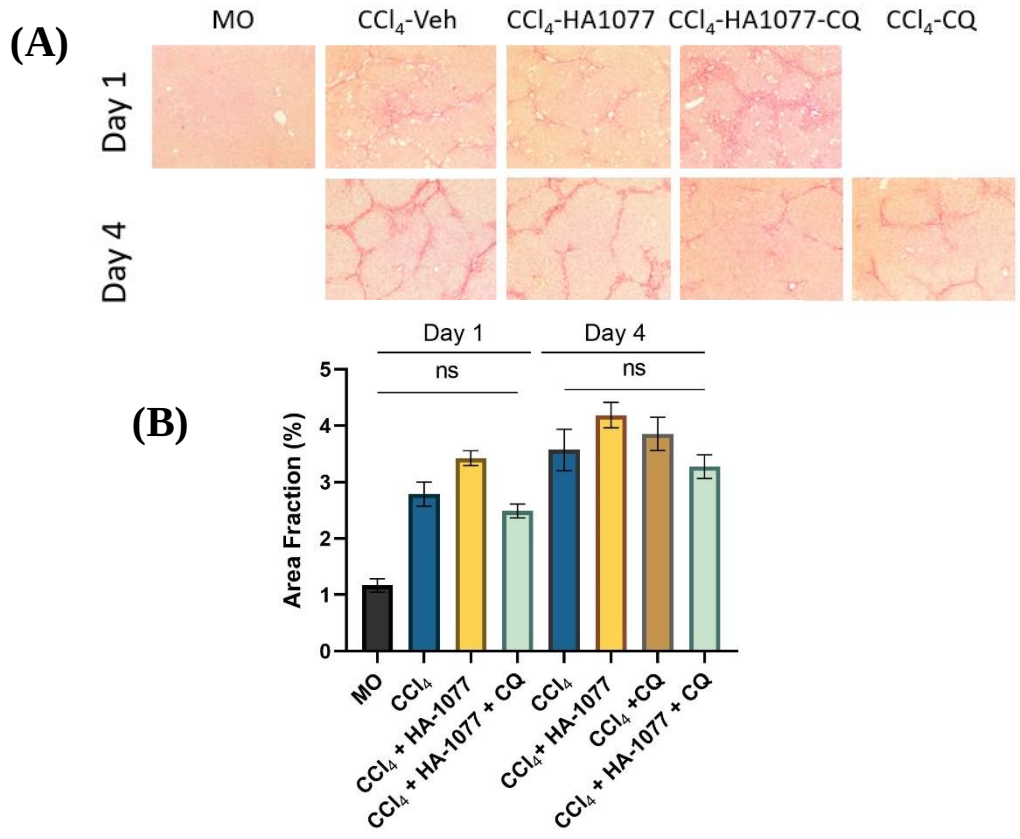

Figure 20. HA-1077 and CQ effect on Sirius red staining of liver section.
(A) Representative images of collagen fibers stained with Picrosirius red in control mineral oil (MO) and treated mice at days 1 and 4 as described previously in Figure 19. Magnification (40X).
(B) Quantification the area of fibrosis in liver. Sirius red staining was quantified using ImageJ and results are expressed as percentage of total area. 8-10 images of the 2-4 lobes were quantified for each mouse. Data are expressed as mean ± SEM; ns not significant (One Way ANOVA).

c. Gene Expression of Hepatic Fibrotic Gene in Response to HA-1077 and CQ in a CCl_4 Regression of Fibrosis.

The effect of HA-1077 and CQ on the expression of the profibrotic gene α-SMA was assessed using RT-PCR. No significant decrease of α-SMA was detected in CCl_4 + HA-1077 and CCl_4 + HA-1077 + CQ compared to CCl_4-treated group (Figure 21).

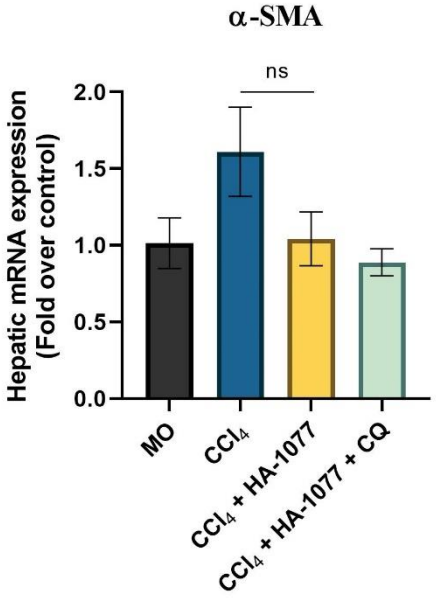

Figure 21. Expression of α-SMA in control and treated groups
Data are shown as minimum to mean ± SEM (n=2 MO, n=8 CCl_4, n=8 CCl_4+HA-1077, n=7 CCl_4+HA-1077+CQ); ns not significant (Mann-Whitney U test)

CHAPTER V

DISCUSSION

Liver fibrosis causes a high morbidity and mortality worldwide regardless of the etiology. Liver injury followed by inflammation and imbalanced deposition of ECM develops into fibrosis and then leads to cirrhosis. It has been demonstrated that liver fibrosis is reversible, and that the resolution of liver injury is promising to assure the resolution of liver function when the cause of injury is removed. Although many ongoing studies aims to address anti-fibrotic strategies, accelerating the regression of liver fibrosis is considered as one of the interesting strategies to resolve liver fibrosis.[71]

It has been previously demonstrated that MJN110 has an anti-fibrotic and anti-inflammatory effect promoting regression of liver fibrosis. Moreover, MJN110 accelerated the regression of liver fibrosis.[104]

To further investigate the mechanisms involved in MJN110-dependent acceleration of fibrosis regression, previous experiments in the lab demonstrated treating mice with the PPAR-ɣ antagonist, GW9662, which resulted in the absence of the acceleration of the fibrosis regression at day 3-4 after the cessation of CCl_4.

In our study, we were not able to see a clear effect of MJN110 on fibrosis as shown in the collagen formation detected by Sirius red staining. One explanation is the variability between the in-vivo experiments where no effect was observed at day 3. Additional treatment days would have shown probably an effect of MJN110. On the gene expression, the results of TGF-β showed a tendency to reverse fibrosis by GW9662. In these experiments, the effect of clodronate liposomes affected both the

effect of MJN110 alone and GW9662, suggesting that macrophages of both pro-fibrotic and anti-fibrotic.

For the inflammatory genes, we found that clodronate liposomes, when administered with MJN110 and GW9662, established an anti-inflammatory effect by significantly decreasing the expression of inflammatory mediators CCL3 and CCL4.

The main interesting result in this model is the MMP13 gene expression. MJN110 treatment induced gene expression of MMP13, a matrix metalloproteinase important in the regression of liver fibrosis and the wound healing of fibrosis. GW9662 blocked the effect of MJN110. Pretreatment of mice with clodronate that are described to uptake by phagocytic restorative macrophages prevented the effects of MJN110 suggesting that the MMP13 can come in part from macrophages. The effect of GW9662 on MJN110 in the presence of clodronate showed an additional effect on MMP13.

This shows that macrophages are major players in inflammation and fibrosis of liver. The major limit of the present experimental results is that clodronate liposomes depleted all types of macrophages. A more selective approach to assess the selective role of macrophage and PPAR-ɣ is to test the effect of MJN110 on selected targeted knockout of PPAR-ɣ in macrophages.

It is important to note, that in these settings, in parallel separate experiments, clodronate did not affect the CCl_4 effects at days 1 and 4.

In parallel, we studied the effect of pioglitazone, a synthetic PPAR-ɣ agonist, on the regression of liver fibrosis. Our results show no acceleration in regression as shown in the Sirius red staining. A significant increase in the expression of the pro-fibrotic gene TGF-β was detected. Controversial effects of PPAR-ɣ-dependent regulation were described in liver fibrogenesis and fibrosis. Pioglitazone-treated mice showed an

absence of modification of hepatic fibrosis of CCl_4-induced model of fibrosis.[112] However, studies in rats using pioglitazone or other PPAR-ɣ agonists have shown an attenuation of liver fibrosis mainly through the inhibition of HSC activation and proliferation.[113, 114] These different outcomes may be caused by the animal model variations and differential expression of PPAR-ɣ.[115] PPAR-ɣ is expressed in different hepatic and immune cells and can trigger different responses.[116] Regulation of PPAR-ɣ in different cells might exert opposing effects and thus cancelling the effect of each other leading to no acceleration in regression. This might explain the results we obtained. Another possibility is that the period of treatment of pioglitazone was not enough to demonstrate an effect. A study showed that pioglitazone did not exert an anti-fibrotic effect when administered in a late stage after five weeks of CCl_4-induced fibrosis in rats (after establishing liver fibrosis) in opposition to its effect when administered early after two weeks of CCl_4 (at the onset of liver fibrosis development in rats).[117] Therefore, we suggest studying the effect of pioglitazone for a longer period of time or to have selective and targeted knockout of PPAR-ɣ in hepatocytes, HSC or macrophages.

In the other study, HA-1077, a ROCK inhibitor, and chloroquine (CQ), an autophagy inhibitor, were used to study whether autophagy is involved in accelerating the regression of liver fibrosis. Our results show that HA-1077 did not accelerate the regression as shown in the Sirius red results (Figure 20). Previous study performed in the lab showed acceleration in regression (The Anti-inflammatory Effect of Rho-Kinase Inhibitors on Liver Fibrosis Progression and Regression In-vivo, Duaa Yaser Hatem, unpublished data). Therefore, we will repeat the quantification of these samples assessing the expression of α-SMA in the HSCs.

Moreover, CQ is a non-selective inhibitor of autophagy of different cells that impairs autophagosome-lysosome fusion.[118] In our results, CQ did not significantly decrease the collagen deposition, as well as the mRNA expression of α-SMA. We suppose that acceleration in regression of liver fibrosis does not involve autophagy. CQ was recently used in an acute model of liver injury where it improved liver function through the decreased expression of liver injury markers of CCl_4-induced liver injury.[119] Moreover, a recent article shows the dual effect of autophagy in liver fibrosis where it can either promote or inhibit fibrosis progression depending on the mediated pathway or the effector cells.[120] Therefore, involvement of autophagy in regression of fibrosis is still debatable and we shall re-evaluate the effect of CQ either using a longer period of treatment or different dosage, or through using selective and targeted knockout of autophagy, like ATG5-LysCRE mice for macrophage autophagy or ATG5-Albumin knockout mice for hepatocyte lacking autophagy to investigate the role of autophagy in the ROCK-kinase-dependent acceleration of regression of liver fibrosis.

As an overall summary, the following figures illustrate the potential effects of different drugs on the different liver cells during liver fibrosis regression (Figure 22, Figure 23).

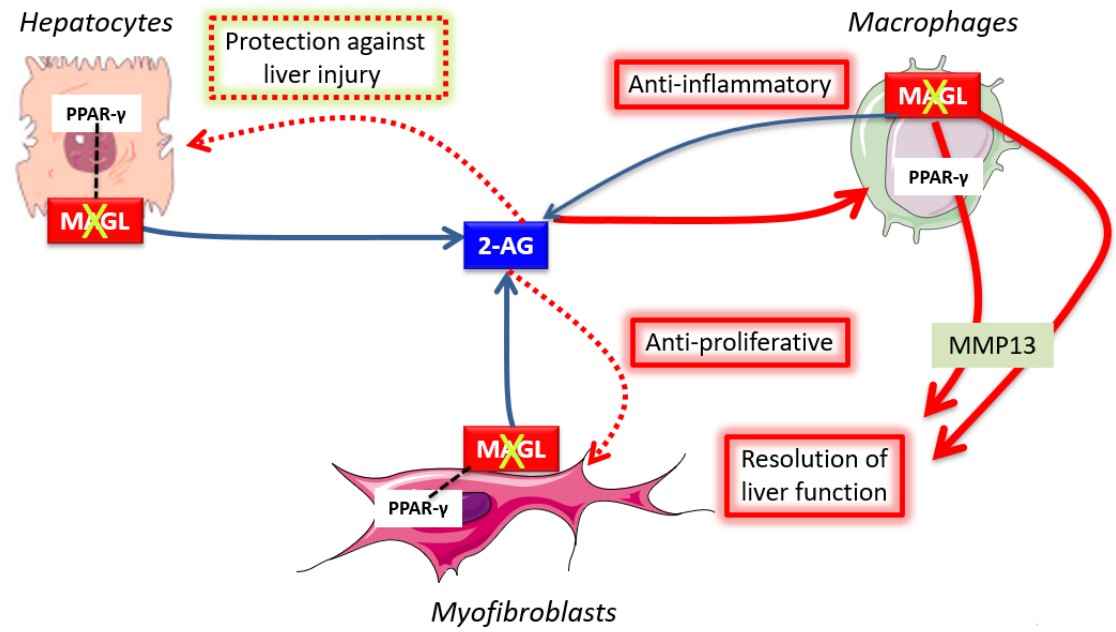

Figure 22. A Scheme Showing the Effect of MJN110 on Different Liver Cells in Relation with PPAR-γ During Fibrosis Regression

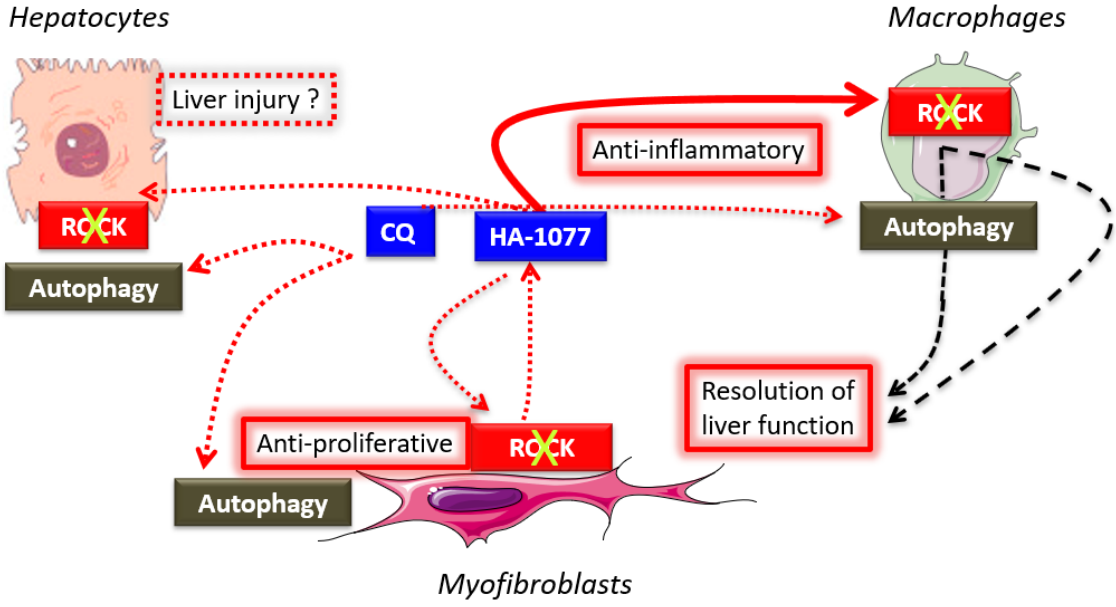

Figure 23. A Scheme Showing the Effect of HA-1077 and CQ on Different Liver Cells During Fibrosis Regression

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