AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF STATIN ON INFLAMMATION AND CHRONIC LIVER FIBROSIS *IN VIVO*

by
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AMERICAN UNIVERSITY OF BEIRUT

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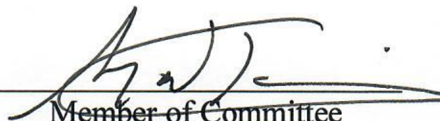

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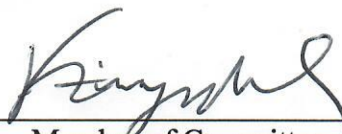

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AN ABSTRACT OF THE THESIS OF

Rola Wafik Al-Sayegh for Master of Science
Major: Biochemistry

Title: Effect of Statins on Inflammation and Chronic Liver Fibrosis *In Vivo*

Background: Persistent hepatic inflammation is deleterious to the liver, as it promotes apoptosis of parenchymal cells and the accumulation of extracellular matrix proteins upon chronic injury. Macrophages, which consist of resident Kupffer cells and monocyte-derived macrophages, are key regulators of liver fibrosis progression and regression in chronic liver diseases. Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase. In addition to inhibiting cholesterol biosynthesis in the liver, statins decrease isoprenoid-dependent modifications of many proteins. They thus mediate pleiotropic beneficial effects such as antioxidant and anti-inflammatory effects.

Aims: In this study, we aim to assess the effect of pitavastatin in liver fibrosis and resolution. We will determine whether pitavastatin has an antifibrotic effect and whether it accelerates the regression of liver fibrosis. We will also investigate if these effects are modulated through the inflammatory pathway.

Methods: Chronic liver fibrosis was induced by injecting 0.6 ml/kg of carbon tetrachloride (CCl_4) intraperitoneally (i.p.) twice a week for 4 or 6 weeks into 10-week-old C57BL/6 male mice. Pitavastatin (10 mg/kg, i.p.) was administered daily. Fibrosis was assessed by Sirius Red staining of collagen fibers, by alpha-smooth muscle actin detection, and gene expression of some fibrotic genes. Moreover, interleukin-6 was assessed at the gene and protein levels. Finally, flow cytometry of the intrahepatic leukocytes was performed.

Results: After 2 weeks of pitavastatin, CCl_4-injected mice exhibited less fibrosis, Sirius red staining decreased by 33% ($p<0.001$, Mann-Whitney U), and α-SMA by 35% ($p<0.05$). Gene expression of fibrotic and inflammatory genes was also decreased compared to mineral oil-injected mice (mRNA levels of fibrotic genes, TGF-β 44%, α-SMA 37% $p<0.05$, and MMP-9 56%, $p=0.08$; and inflammatory genes, IL-6 mRNA levels 59%, $p<0.05$). When assessing the effect of statins on fibrosis regression, pitavastatin decreased fibrosis (Sirius red between 21% and 31%, $p< 0.005$, α-SMA protein levels 34% $p<0.001$ and gene expression of PDGFRβ by 28%, α-SMA by 35% and MMP-2 by 38% $p<0.05$) suggesting a possible acceleration of the regression process. Moreover, pitavastatin diminished inflammation during the regression experiment by reducing the proinflammatory macrophages ($Ly6C^{high}$ cells, 17% and 8% for days 2 and 3, respectively) and shifting towards restorative macrophages ($Ly6C^{low}$ cells increased by 56% and 53% for days 2 and 3, respectively).

Conclusion: Overall, our results demonstrate that pitavastatin attenuates the development of liver fibrosis, and accelerates its regression *in vivo*, probably through modulating inflammation. Additional experiments are required to decipher the mechanism by which pitavastatin modulates both liver regression and fibrosis.

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ABBREVIATIONS

ALT	alanine aminotransferase
AST	aspartate aminotransferase
BDL	bile duct ligation
BMDM	bone marrow-derived macrophages
CCL2	chemokine (c-c motif) ligand 2
CCl_3	trichloromethyl radical
CCl_4	carbon tetrachloride
CCL5	chemokine (c-c) ligand 5
CCR2	chemokine (c-c motif) receptor 2
CD11b	cluster of differentiation molecule 11b
CDAA	choline-deficient, L-amino acid deficient
cDNA	complementary DNA
CLDs	chronic liver diseases
COX-2	cyclooxygenase-2
CTGF	connective tissue growth factor
CXCL16	chemokine (c-x-c motif) ligand 16
CXCR6	c-x-c chemokine receptor type 6
CYP2E1	cytochrome P450 2E1
DAMPs	damage-associated molecular patterns

DCs	dendritic cells
DEN	diethylnitrosamine
DMS	dimethylnitrosamine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribnuclease
DTR	diphtheria toxin receptor
DTT	dithiothreitol
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
FACS	fluorescence-activated cell sorting
FAO	fatty acid oxidation
FLT3L	FMS-like kinases-3 ligand
HBSS	Hank's balanced salt solution
HBV	hepatitis b virus
HCV	hepatitis c virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFD	high-fat diet
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMGB1	high-mobility group protein box 1

HSCs	hepatic stellate cells
HVI	hepatitis virus infection
i.p.	intraperitoneal
IACUC	institutional animal care and use committee
IFN-γ	interferon-gamma
IGF-1	insulin-like growth factor-1
IL-1β	interlukin-1 beta
IL-6	interlukin-6
KCs	Kupffer cells
KLF2	Kruppel-like factor 2
LDL	low-density lipoprotein
LPS	lipopolysaccharide
MCD	methionine-and choline deficient
mg/kg	milligram per kilogram
min	minutes
ml/kg	milliliter per kilogram
MMPs	matrix metalloproteinases
MO	mineral oil
mPGES-1	microsomal prostaglandin synthase-1
NaCl	sodium chloride
NaF	sodium fluoride

NAFLD	non-alcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NGF	nerve growth factor
NK	natural killer cells
NKG2D	natural killer group 2d
NO	nitric oxide
ns	not significant
NTPDase 2	ectonucleotidase 2
PAMPs	pathogen-associated molecular patterns
PBC	primary biliary cirrhosis
PBS	phosphate-buffered saline
PDGF	platelet-derived tissue growth factor
PDGFRβ	platelet-derived tissue growth factor receptor beta
PFs	portal fibroblasts
PGE2	prostaglandin E2
PPARa	peroxisome proliferator-activated receptor alpha
PRRs	pattern recognition receptors
PSC	primary sclerosing cirrhosis
RNA	ribonucleic acid
RNase	ribonuclease
ROCK	rho-associated protein kinase

ROS	reactive oxygen species
RT-PCR	real-time polymerase chain reaction
SEM	standard mean of error
STAT3	signal transducer and activator of transcription 3
TAA	thioacetamide
TGF-β	transforming growth factor beta
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
TIMPs	tissue inhibitor of metalloproteinase
TLRs	toll-like receptors
TNF-α	tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	regulatory t cells
α-SMA	alpha smooth muscle actin
μg	microgram
μl	microliter
μm	micrometer

CHAPTER I

INTRODUCTION

A. Liver Fibrosis

Chronic Liver Diseases (CLDs) are a major and increasing cause of morbidity and mortality with worldwide estimations showing 844 million people with CLDs, and a mortality rate of 2 million deaths per year (Marcellin & Kutala, 2018). Liver fibrosis, one of several CLDs, is a wound healing response to chronic liver damage that possesses various etiologies such as viral infection, alcohol abuse, and injuries of the autoimmune, toxic or metabolic types.

1. Factors that Predispose Humans to Hepatic Fibrosis

In the liver, genetic alterations, metabolic disorders, cholestasis, viral infections, parasites, drugs, toxins, alcohol, and a broad variety of other noxious and environmental factors can lead to the initiation and progression of hepatic fibrosis (Figure 1) (Weiskirchen, Weiskirchen, & Tacke, 2018b).

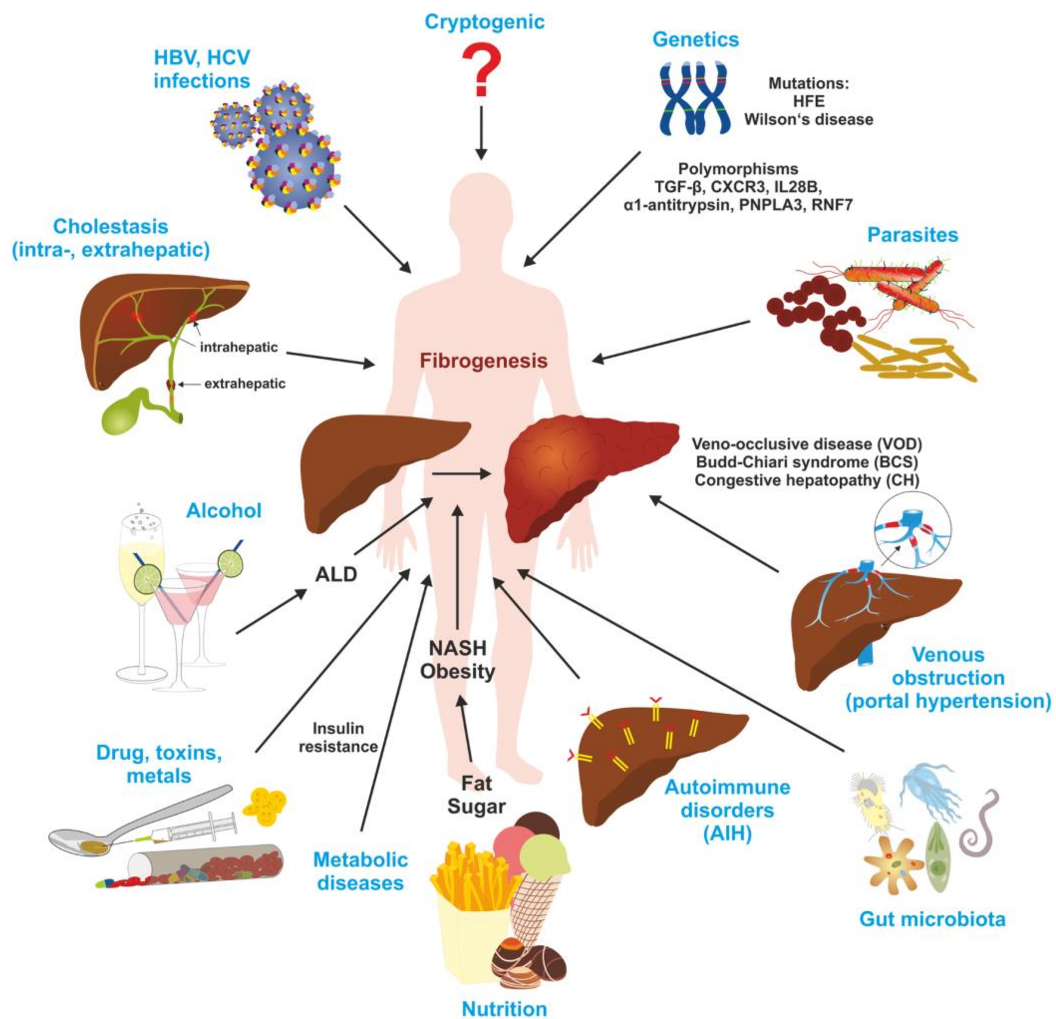


Figure 1. **Major causes of liver fibrosis** adapted from (Weiskirchen et al., 2018b).

a. <u>Alcohol</u>

Excessive alcohol consumption is the leading cause of liver disease that affects millions of people worldwide (Kawaratani et al., 2017). Alcohol liver disease pathogenesis involves oxidative stress, acetaldehyde adducts, abnormal methionine metabolism,

endotoxin activation, impaired hepatic regeneration, and the metabolism of alcohol into toxic products (Haber, Warner, Seth, Gorrell, & McCaughan, 2003). Alcohol is metabolized in the liver by the enzymes cytochrome P450 2E1 (CYP2E1) and alcohol dehydrogenase to produce acetylaldehyde (Garcin et al., 1985; Neve & Ingelman-Sundberg, 2000). The product has a stronger toxicity than ethanol and can lead to liver injury which triggers the production of reactive oxygen species (ROS) and generates lipid peroxidation products (Wu & Cederbaum, 2005). Acetylaldehyde directly upregulates the transcription of collagen I and triggers the synthesis of transforming growth factor beta (TGF-β) (Purohit & Brenner, 2006). Furthermore, upon ingesting alcohol, the permeability of the intestinal membrane is augmented and the portal blood endotoxin (e.g. lipopolysaccharide LPS) concentration continuously increases (Rao, Seth, & Sheth, 2004). Ultimately, chronic alcohol abuse leads to liver damage, liver inflammation, fibrosis, cirrhosis and hepatocellular carcinoma (Kawaratani et al., 2017).

b. Hepatitis Virus Infection

Infections with hepatitis B and C viruses are a global health problem that cause CLDs and are the leading motive for liver transplantation worldwide. Chronic hepatitis B or C are associated with significant mortality and account for more than 1.3 million deaths per year (Petruzziello, 2018).

The hepatitis B virus (HBV) is a partially double-stranded DNA virus that replicates *via* reverse transcription in the hepatocytes (Trepo, Chan, & Lok, 2014). A small number of the HBV virion (1-10) is sufficient to initiate infection, and the natural history of chronic HBV infection is classified in specific stages that are defined by hepatic inflammation activity

and viral replication rate (Chen & Yang, 2011; Trepo et al., 2014). On the other hand, the hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus that replicates in the cytoplasm of the hepatocytes (Ringelhan, McKeating, & Protzer, 2017). From a structural point of view, HCV circulates in the blood as lipoviral particles, where viral entry into the hepatocytes, replication, and assembly are dependent on host lipid metabolism and take place in lipid droplets (Popescu et al., 2014; Schaefer & Chung, 2013). Of note, geranylgeranyl, a cholesterol intermediate, is an essential component of HCV replication *in vitro* (Kapadia & Chisari, 2005). The mechanisms underlying HCV/HBV-infection are mainly: (1) increased hepatocyte proliferation and steatosis, (2) induced inflammation and oxidative stress, (3) mitochondrial damage and induction of ROS and (4) virus-induced host immune response (Chen & Yang, 2011; Ringelhan et al., 2017).

c. Nutrition and Metabolic Diseases

Obesity and its multiple metabolic ramifications, including type 2 diabetes mellitus, heart diseases, and fatty liver diseases, are advancing worldwide and especially in western countries (Brunt, 2010). Excess fat and other lipotoxic mediators that stimulate oxidative stress, alteration of hepatic mitochondrial β-oxidation, and modifications in the microbiota composition of the gastrointestinal tract are associated with non-alcoholic fatty liver disease (NAFLD) and, eventually, the initiation and progression of hepatic fibrosis leading to nonalcoholic steatohepatitis (NASH) (Arab, Arrese, & Trauner, 2018). Additionally, recent evidence suggests that sugar-enriched diets are among the culprits in the pathogenesis of NAFLD, as they affect gut microbiota and trigger hepatic fat accumulation due to the impairment of fat oxidation (Lambertz, Weiskirchen, Landert, &

Weiskirchen, 2017). Hepatic fatty-acid overload results in ROS production, tumor necrosis factor alpha (TNF-α) signaling, and apoptosis of hepatocytes (Ribeiro et al., 2004). Evidence revealed that, as a consequence of lipid peroxidation metabolites and ROS generation, as well as the permanent inflammatory response and increase in TGF-β expression, hepatic stellate cells (HSCs) become directly activated to produce scar-forming collagen, thereby leading to liver fibrogenesis (Liedtke et al., 2013).

d. Cholestasis

Cholestatic liver disease results from the disruption of proper bile secretion, synthesis, and/or flow through the biliary tract. Hepatic production of bile occurs in the liver *via* the secretion of bile salts and electrolytes from hepatocytes into bile canaliculi (Pollock & Minuk, 2017). The bile secretory unit is sealed by junctional complexes to prevent the reversal of the bile flow into the liver that triggers hepatocyte apoptosis, owing to the potential cytotoxic nature of bile salts (Boyer, 2013; Faubion et al., 1999). Multiple causes of bile duct injury have been described. These include autoimmune diseases, termed Primary Biliary Cirrhosis (PBC) and Primary Sclerosing Cirrhosis (PSC), obstruction conditions, toxic injury, and loss-of function mutations such as those affecting JAG1 or NOTCH2 genes (McDaniell et al., 2006; Pollock & Minuk, 2017). As bile acids accumulates in the liver, they act as strong detergents that cause unspecific cellular damage and initiate a cascade of inflammatory and fibrogenic events *in situ*, thus inducing hepatic fibrosis (Pollock & Minuk, 2017; Rao et al., 2004).

However, irrespective of the underlying noxious trigger, liver damage induces an inflammatory response leading to the activation of matrix-producing cell populations and deposition of extracellular matrix (ECM) proteins in the tissue.

2. *Pathogenesis of Liver Fibrosis*

Hepatic fibrosis is characterized by the formation and excessive accumulation of fibrous connective tissue, leading to architectural tissue remodeling (Weiskirchen, Weiskirchen, & Tacke, 2018a). The fibrogenic process results from the combination of increased synthesis and deposition of ECM proteins (collagen, glycosaminoglycans, laminin, elastin and proteoglycans) within the liver and a parallel alteration of matrix degradation mechanisms (Lotersztajn, Julien, Teixeira-Clerc, Grenard, & Mallat, 2005).

Upon scarring, the normal composition of the ECM in the perisinusoidal space is replaced by nonfunctional matrix rich in fibrillar collagen I and III, building a network that is resistant to matrix metalloproteases (MMPs) system. MMPs, a group of endopeptidases, are the chief degrading effectors which are capable of degrading a broad range of ECM components (Ramachandran & Iredale, 2009). MMPs play major roles in tissue development, matrix turnover, repair, and remodeling. Their proteolytic activity is usually regulated by tissue inhibitors of metalloproteinases (TIMPs) by binding tightly to their catalytic sites. Failure of matrix degradation brought on by the inhibition of MMP degradative activity, and the abundant synthesis of ECM proteins by hepatic myofibroblasts, causes progressive fibrosis. (Campana & Iredale, 2017).

The pathogenic sequence of fibrogenesis is initiated by hepatocyte destruction *via* necrosis or apoptosis, both of which are associated with the release of cellular components

such as danger-associated molecular patterns and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) (Brenner, Galluzzi, Kepp, & Kroemer, 2013). As fibrosis progresses, hepatocytes begin to regenerate by forming nodules that will further modify the architecture of the liver leading to a progressive loss of its function (Friedman, 1993).

Moreover, advanced hepatic fibrosis results in cirrhosis, portal hypertension, hepatic carcinoma with the loss of the internal hepatic architecture, and eventually liver failure (Figure 2) (Lotersztajn et al., 2005; Pellicoro, Ramachandran, Iredale, & Fallowfield, 2014).

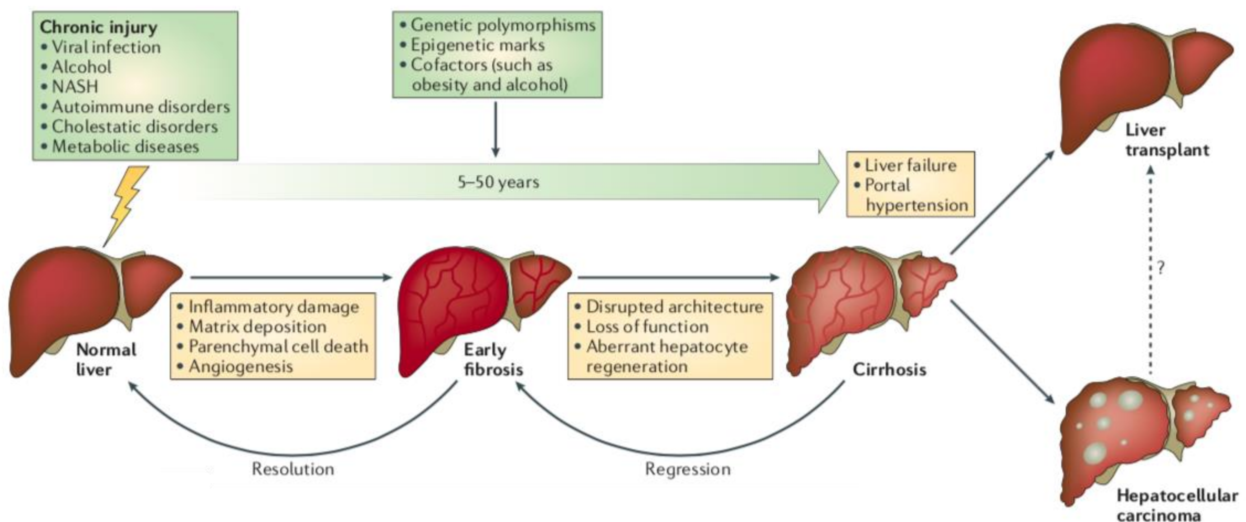


Figure 2. **Progression of liver fibrosis** adapted from (Pellicoro et al., 2014).

3. *Cellular Effectors in Liver Fibrosis*

Liver cells are divided into parenchymal and non-parenchymal cells, with the former group consisting of hepatocytes, and the latter group consisting of resident macrophages, HSCs, endothelial cells, and others. ECM accumulation during injury is driven by a heterogeneous population of myofibroblasts that migrate and accumulate at sites of liver injury (Mallat & Lotersztajn, 2013). In the injured tissue, these cells are implicated in wound healing by extracellular collagen deposition and contracting the matrix to seal an open wound (Weiskirchen et al., 2018a). However, as the injury progresses, hepatic myofibroblasts fail to undergo apoptosis resulting in the persistence of these cells in the liver. Studies have shown that hepatic myofibroblasts are of diverse origins and coexist in the injured liver, with the HSCs being the most abundant and the resident portal fibroblasts making up a minor portion of the injured liver (Lotersztajn et al., 2005).

a. Hepatic Stellate Cells

HSCs, also known as the Ito cells, are found in the perisinusoidal space between hepatocytes and sinusoidal endothelial cells of the liver, and store vitamin A in the form of retinol esters (Mallat et al., 2014). In a normal liver, HSCs exist in their quiescent state and represent 5-8% of the total number of liver cells (Lepreux & Desmouliere, 2015; Lotersztajn et al., 2005).

Upon liver injury, HSCs transdifferentiate into alpha smooth muscle actin (α-SMA)-expressing myofibroblasts, start to proliferate, and give rise to 82-96% of the hepatic myofibroblast pool (Mederacke et al., 2013). These hepatic myofibroblasts develop contractile function, secrete large amounts of ECM degrading enzymes such as MMP-2 and

MMP-9, and alter ECM degradation mechanism *via* TIMP expression (Campana & Iredale, 2017). Moreover, myofibroblasts secrete profibrogenic and proinflammatory agents such TGF-β and interleukin-6 (IL-6), respectively (Figure 3) (Berenguer & Schuppan, 2013; Carloni, Luong, & Rombouts, 2014). Other important profibrogenic mediators include connective tissue growth factor (CTGF) and members of the platelet-derived tissue growth factor (PDGF) family, the latter being potent mitogens for the myofibroblasts driving their proliferation at the site of injury (Borkham-Kamphorst & Weiskirchen, 2016). Additionally, myofibroblasts become resistant to apoptosis upon engulfing the apoptotic bodies that are derived from the death of hepatocytes (Jiang et al., 2010).

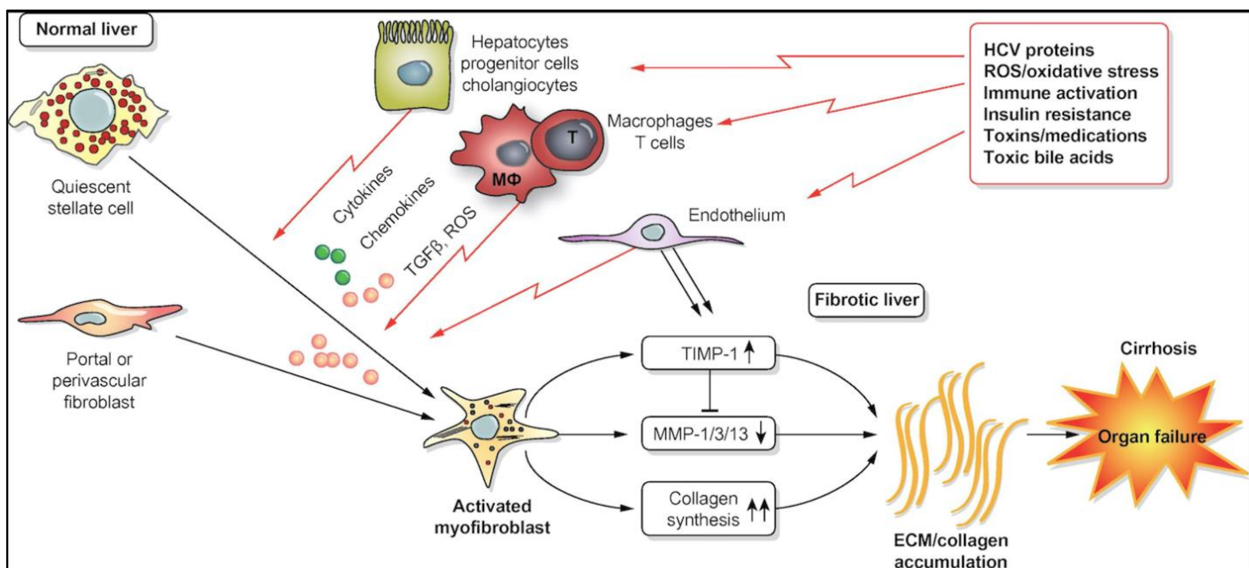


Figure 3. **Mechanism of hepatic fibrosis** adapted from (Berenguer & Schuppan, 2013).

b. Portal Fibroblasts

Portal fibroblasts (PFs), located around the portal vein, comprise a small population of fibroblastic cells (Karin, Koyama, Brenner, & Kisseleva, 2016). Recent studies have shown their involvement in the pathogenesis of cholestatic liver fibrosis (El Mourabit, Loeuillard, Lemoinne, Cadoret, & Housset, 2016). Similar to HSCs, PFs get activated in response to chronic injury, transforming into myofibroblasts that express α-SMA and synthesize ECM proteins (Dranoff & Wells, 2010). Considering that PFs lose their distinguishing markers, such as ectonucleotidase 2 (NTPDase 2), upon activation, the origin of the myofibroblasts, and the kinetics of activation and proliferation of HSCs and PFs is still unclear (Dranoff et al., 2004).

4. *Model of Chronic Liver Injury Induced by Carbon Tetrachloride (CCl_4)*

Experimental liver fibrosis in rodents is presently the gold standard to confirm a proposed disease-associated mechanism that closely mimics clinical situations. In particular, the single or repeated administration of CCl_4 has become one of the most commonly used approaches to induce toxin-mediated liver fibrosis (Scholten, Trebicka, Liedtke, & Weiskirchen, 2015). In mice, typically 0.5 to 2 ml/kg body weight CCl_4 is injected intraperitoneally (i.p.) two to three times per week, resulting in vigorous and highly reproducible liver fibrosis between 4 and 6 weeks of treatment (Liedtke et al., 2013). CCl_4 is metabolized by the cytochrome P450 enzymes in the hepatocytes, forming the toxic free radical trichloromethyl (CCl_3) which further causes hepatocyte necrosis (Slater, Cheeseman, & Ingold, 1985). Subsequently, this free radical provokes liver injury by the production of ROS, and the peroxidation of lipids, proteins and DNA (Scholten et al.,

2015). For many years, experimental studies have demonstrated that repeated doses of CCl_4 lead to repeated rounds of wound healing, causing HSC activation, imbalance between ECM production and degradation, and development of progressive hepatic fibrosis (Delire, Starkel, & Leclercq, 2015; Friedman, 2000; Starkel & Leclercq, 2011). Moreover, CCl_4-mediated liver fibrosis is characterized by the activation of resident macrophages that release cytokines, chemokines and other proinflammatory factors, thus initiating an inflammatory response (Heindryckx, Colle, & Van Vlierberghe, 2009). Of note, CCl_4-induced liver fibrosis in mice can be completely resolved within several weeks after withdrawal of the toxic treatment (Kisseleva et al., 2012).

Thus, the CCl_4 model exhibits all the important properties of human liver fibrosis, including inflammation, regeneration, fiber formation and potential fibrosis regression. Other models of chronic liver injury are summarized in Table 1 adapted from (Delire et al., 2015; Heindryckx et al., 2009).

Liver fibrosis induced animal model		Main features
Hepatotoxin-induced liver fibrosis	CCl_4	- Significant fibrosis after 4-6 weeks. - Fibrosis reversion in a short time after CCl_4 withdrawal. - Metabolization by cytochrome P450 CYP2E1. - Release of the highly reactive free radical (CCl_3).
	Thioacetamide (TAA)	- Leads to severe fibrosis after 12 to 16 weeks in rats and 16 to 24 weeks in mice. - Liver fibrosis reversion requires more than 2 months after TAA withdrawal. - CYP450 is described as a major contributor in TAA metabolism. - Release of a highly reactive product Sulphur dioxide (S, S-dioxide).
	Dimethylnitrosamine (DMS) and diethylnitrosamine (DEN)	- Used to study the progression from fibrosis to cancer. - Metabolized by CYP2E1 and other P450 isoenzymes. - Toxic activity mediated through DNA-adduct formation.
Biliary fibrosis	Common bile duct ligation model (CBDL)	- Induces obstructive cholestatic injury. - Mice and rats develop fibrotic reaction after 14 to 28 days. - Reversibility is feasible after bilioduodenal anastomosis (duct reconnection)

Alcohol-induced liver disease	Lieber-De Carli liquid diet model	- Administration of an alcohol containing isocalorically-controlled liquid diet as the sole source of food and drink. - Mild steatosis and low-grade inflammation but no significant fibrosis even after prolonged administration.
	Intragastric feeding model by Tsukamoto-French	- Fibrosis development after 6 to 8 weeks. - Sustained high alcohol blood level. - Main limitation: implantation of the intragastric canula.
Non-alcoholic fatty liver disease	High-fat diet (HFD)	- Signs of hepatic inflammation and fibrosis observed after 4 weeks of the diet in rats and 3 months in mice. - Development of Insulin resistance.
	Methionine-and choline deficient diet (MCD)	- Development of steatosis and inflammation after 7 to10 weeks of treatment. - Lack of metabolic features of NASH: no insulin resistance, weight loss, and a decrease in triglyceride and cholesterol levels.
	Choline-deficient, L-amino acid deficient diet (CDAA)	- Development of fibrosis, inflammation and steatosis after 10 weeks of treatment. - Development of insulin resistance, no weight loss, and higher plasma triglycerides compared to MCD diet.
Genetically modified models	Overexpression of TGF- β1, and PDGF-β	- Some require a second hit such as, CCl_4 injection or MCD diet to develop fibrosis, inflammation, or hepatocellular carcinoma.

B. Table 1**. Animal models of liver fibrosis.**

C. Inflammation as a Key Driver of Liver Fibrogenesis

Liver damage initiates an orchestrated repair process intent on preserving organ function and eliminating the initial cause of injury or underlying harmful stimuli. The persistence of chronic inflammation is a hallmark, coupled with progressive hepatic fibrosis and the development of advanced hepatic damage (Gressner & Weiskirchen, 2006). In the early phase of liver damage, dead hepatocytes release DAMPs and PAMPs such as the nuclear protein high-mobility group protein B1 (HMGB1) and LPS respectively, that are recognized by specific pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Heymann & Tacke, 2016). This inflammatory response stimulates the production of ROS and the activation of resident immune cells present in the liver, which includes dendritic cells (DCs), mast cells, macrophages termed Kupffer cells (KCs), and others (Lee & Friedman, 2011; B. J. Park, Lee, & Lee, 2014).

1. Macrophages Response During Liver Damage

Macrophages play a critical role in the innate immune response in the injured liver. Studies from healthy murine livers estimated that the quantity of macrophages was roughly 20 to 40 for every 100 hepatocytes present (Lopez, Tsai, Baratta, Longmuir, & Robertson, 2011). Liver macrophages consist of two distinct populations: (1) resident macrophages (KCs), and (2) blood/bone marrow-derived macrophages that are termed *infiltrating macrophages* (Pellicoro et al., 2014).

KCs are highly effective phagocytes that get activated upon the release of cellular debris, ROS, DAMPs and PAMPs, and ensure liver homeostasis and the eradication of dead

cells (Dini, Pagliara, & Carla, 2002). After activation, KCs release chemokines such as chemokine (C-C motif) ligands 2 and 5 (CCL2 and CCL5) that foster the infiltration of monocytes from circulation into the liver. Consequently, the monocyte-derived macrophages may promote fibrosis by releasing factors such as nitric oxide (NO), TGF-β, IL-1β, PDGF and CCL2 that in turn further activate HSCs and exacerbate inflammation (Figure 4) (Campana & Iredale, 2017). Compelling studies using CCL2 inhibitors or CCL2 knockout animals have shown an attenuation in hepatic macrophage infiltration, inflammation, and subsequently, hepatic fibrosis following chronic injury (Pellicoro, Ramachandran, & Iredale, 2012). Likewise, the importance of macrophages in scar formation was further validated in a study in which macrophages were selectively depleted using a transgenic mouse model with diphtheria toxin receptor expression on myeloid ($CD11b^+$) cells, known as CD11b-DTR transgenic mice, and showed reduced fibrosis in these mice (Duffield et al., 2005).

Over the years, the understanding of the heterogeneity of macrophages in the liver during injury has increased dramatically. In 2012, Iredale et al. first described in a CCl_4-induced fibrosis model the presence of two major populations of macrophages that adapt their phenotype according to the hepatic microenvironment. Infiltrating macrophages expressing Ly6C marker ($Ly6C^{high}$) are profibrogenic and proinflammatory macrophages, while $Ly6C^{low}$ macrophages are restorative, antifibrotic and anti-inflammatory macrophages (Ramachandran et al., 2012) (discussed in detail in section C-4). Therefore, the tight association between inflammation and fibrogenesis suggests that therapies suppressing liver inflammation should also be useful in preventing or reversing hepatic fibrosis.

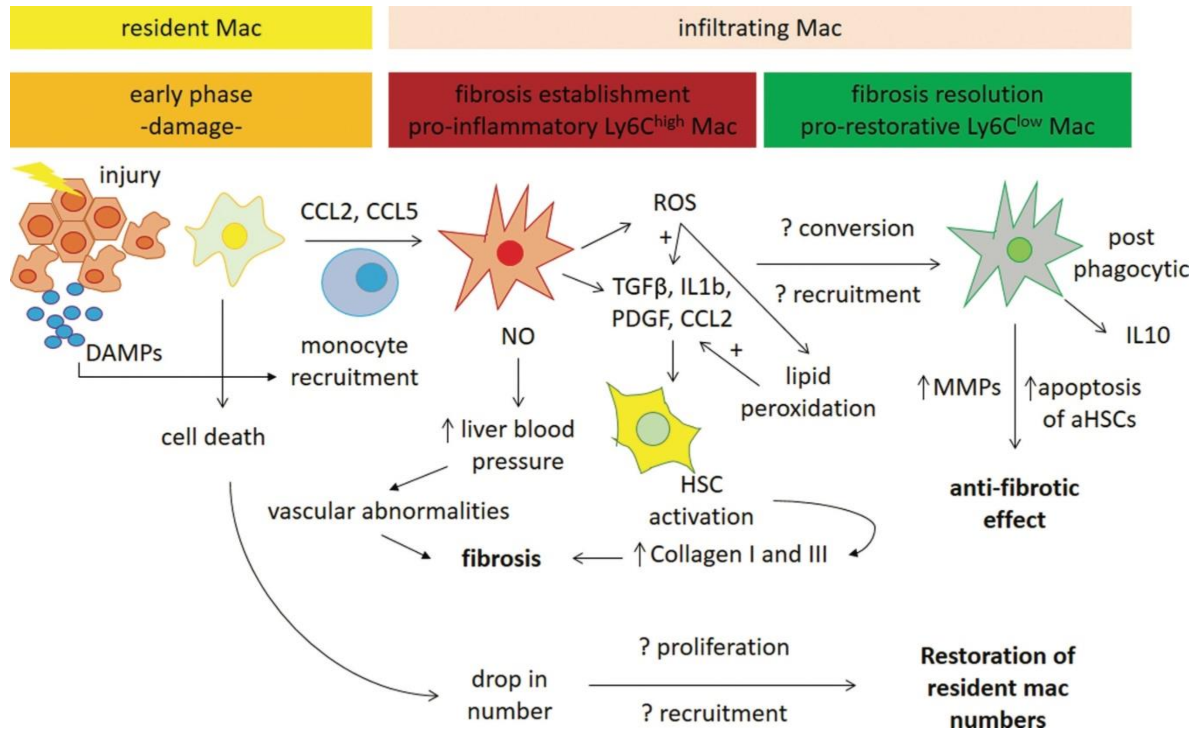


Figure 4. **Macrophage role in the fibrotic response during liver injury** adapted from (Campana & Iredale, 2017).

2. Other Immune Cells

Other immune cells, such as T cells and neutrophils, play a role in chronic liver fibrosis and will be concentrated on in this section.

a. <u>T cells</u>

$CD4^+$ T lymphocytes play a major role in the fibrogenic process, manifesting either a positive or negative outcome depending on their phenotype. Indeed, whereas T helper 2 (Th2) polarization promotes liver fibrosis *via* the production of IL-13, T helper

1(Th1) responses reduce liver fibrogenesis *via* the release of interferon-gamma (IFN-γ) (Pellicoro et al., 2014). IL-13, a typical Th2 cytokine, increases the expression of TGF-β1 in myofibroblasts; this in turn fosters the production of MM-9, which can further convert pro-TGF-β1 into TGF-β1, and perpetrating the profibrotic response (Wynn, 2004). On the other hand, IFN-γ suppresses the deposition of collagen, and cooperates in tipping the balance of MMPs/TIMPs toward the MMPs. Moreover, it blocks the production of the Th2 cytokines. (Muhanna et al., 2008; Wynn, 2004).

T helper 17 (Th17) lymphocytes have also emerged as critical enhancers of the profibrogenic potential of hepatic myofibroblasts *via* the secretion of IL-17 (Rolla et al., 2016). The IL-17 receptor is expressed on numerous cell types and, upon activation, induces the secretion of proinflammatory cytokines such as IL-1β, IL-6, TNF and TGF-β (Korn, Bettelli, Oukka, & Kuchroo, 2009). Additionally, IL-17 controls the synthesis of type I collagen in HSCs through the activation of the signal transducer and activator of transcription 3 (STAT3) signaling pathway (Meng et al., 2012).

The regulatory T (Treg) cells regulate other immune cells in a dominant-negative manner (Pellicoro et al., 2014). In the bile duct ligation (BDL) rat model, the depletion of Treg cells exacerbates fibrosis (Katz et al., 2011). Moreover, Treg cells produce the immunosuppressive cytokine IL-10, thereby ameliorating fibrosis in the BDL model (Pellicoro et al., 2014). However, a subset of Treg that secretes IL-8 was suggested to be profibrotic in chronic HCV infection (Langhans et al., 2013).

The precise role of $CD8^+$ T lymphocytes in the development of hepatic fibrosis is controversial. Studies demonstrated that the adoptive transfer of $CD8^+$ cells can have a profibrogenic role in the liver, while in other studies, mice that were depleted from $CD8^+$ T

cells showed no impact on the development of liver fibrosis in response to CCl_4 compared to control animals (Novobrantseva et al., 2005; Safadi et al., 2004).

b. Neutrophils

Data that supports the role of neutrophils in fibrogenesis is limited. Macrophages recruited *via* activated HSCs attract neutrophils at the site of damage and, in models of liver damage, neutrophils are consistently shown to be early mediators. Indeed, neutrophil depletion in the BDL-model had no discernable effect on fibrosis, despite there being a decrease in the number of hepatic neutrophils (Saito, Bostick, Campe, Xu, & Maher, 2003). However, adoptively transferred macrophages in a model of CCl_4-induced fibrosis augmented the number of recruited neutrophils, which in turn was associated with an increase in MMPs of neutrophil origin and a subsequent antifibrotic effect (Harty et al., 2010). Although, it is controversial whether neutrophils are present in the liver in adequate numbers during the resolution phase to have a meaningful effect.

c. Other Immune cells

Other immune cells have also been involved. Natural killer (NK) cells reduce hepatic fibrosis and mediate the direct killing of senescent HSCs. NK cells also exert an antifibrotic role by the production of cytokines such as IFN-γ that induce HSC apoptosis (Radaeva et al., 2006). Natural-killer-T-cells, on the other hand, have a profibrotic effect through the production of IL-4, regulated by the CXCR6/CXCL16 axis (Geissmann et al., 2005). Moreover, DCs are innate immune cells that may orchestrate the inflammatory response during both progression and resolution of liver fibrosis (Connolly et al., 2009;

Henning et al., 2013). Additionally, the depletion of B cells in a model of chronic CCl_4 intoxication showed beneficial effects on liver fibrosis. It could be speculated that B cells have a profibrotic role (Novobrantseva et al., 2005).

D. Regression of Liver Fibrosis

Liver fibrosis regression is the process of matrix degradation and restoration of hepatocyte mass to reestablish normal liver histology and function (Iredale & Bataller, 2014). Although traditionally seen as an irreversible process, compelling evidence from rodent models and human studies indicated that advanced fibrosis, even at the cirrhotic stage, may regress following the control of the noxious stimuli (Jun & Lau, 2018). Iredale et al. showed in a model of CCl_4-induced fibrosis that cessation of dosing is followed by the reversal of fibrosis within four weeks through myofibroblast apoptosis and reduced expression of metalloproteinase inhibitors (Iredale et al., 1998). Similarly, fibrosis evoked by BDL or viral infection resolved following biliojejunal anastomosis and suppression of virus replication, respectively (Ellis & Mann, 2012).

The hepatic capacity to remodel scar tissue follows specific mechanistic principles that include the termination of chronic tissue damage, shifting the cellular bias from inflammation to resolution, myofibroblast apoptosis and deactivation, and finally, fibrinolysis of excess ECM proteins (Weiskirchen et al., 2018a).

1. Arrest of Chronic Liver Damage

Studies showed that, during hepatic fibrogenesis, several restorative mechanisms are induced during injury, but all these mechanisms are not sufficient for preventing ongoing fibrogenesis in the presence of persisting injury (Weiskirchen & Tacke, 2016). Fibrosis arrest is best achieved by successful removal of the causative agent (hepatotoxins or diet) (Baeck et al., 2014). The termination of the underlying cause of tissue damage promotes regenerative pathways in parenchymal cells and hinders any further activation of myofibroblasts (Cordero-Espinoza & Huch, 2018). Additionally, the release of proinflammatory endogenous danger signals, such as HMGB1, or the release of free DNA into the extracellular milieu during states of cellular stress or damage, are terminated upon cessation of liver damage (Luedde, Kaplowitz, & Schwabe, 2014).

2. Myofibroblasts Deactivation and Elimination

The reduction or inactivation of cells that are causative for the extensive ECM production remains to be a challenge during the regression of fibrosis. As mentioned previously, HSCs are the main collagen-producing cells in the liver that transdifferentiate into myofibroblasts. In general, there are a number of possibilities for how the liver can facilitate the clearance or inactivation of these harmful cells. Activated fibrogenic myofibroblasts can undergo apoptosis, revert to an inactive/quiescent phenotype, or become senescent cells (irreversible cell cycle arrest) (Jun & Lau, 2018).

Studies have illustrated, using *in vitro* and *in vivo* models of fibrosis regression, that activated HSCs are removed both by apoptosis and by phenotypic reversion to quiescent state (Pellicoro et al., 2012). Wright et al. have shown that inducing apoptosis by

gliotoxin in a model of chronic CCl_4 administration leads to a reduction in fibrosis (Wright et al., 2001). There are a multitude of soluble factors, including growth factors (NGF, IGF-1, TGF-β) and death receptor ligands (TRAIL, Fas), that provoke the initiation of hepatic myofibroblast apoptosis (Elsharkawy, Oakley, & Mann, 2005). Interestingly, the physical characteristics of the environment of the myofibroblasts have important effects on cell survival. Specifically, myofibroblast apoptosis is itself dependent on the presence of active collagen I, as evidenced by the critically impaired HSC apoptosis among transgenic mice that express a non-degradable form of collagen I, resulting in a failure to spontaneously remodel hepatic scars and a persistence of activated myofibroblasts following the cessation of CCl_4 injury (Issa et al., 2003). Moreover, investigators have demonstrated using genetic tracking experiments for activated HSCs that phenotypically quiescent, but previously activated, HSCs can be found 30 to 45 days after withdrawal of CCl_4 (Troeger et al., 2012). Similarly, Kisseleva et al. have shown using, Cre-loxP-based genetic labeling of hepatic myofibroblasts, that activated HSCs were reverted to a quiescent-like phenotype seven days after termination of the CCl_4 dosage (Kisseleva et al., 2012).

Another option for clearance of the activated HSCs is the senescence of these cells. Studies have shown that senescent cells accumulate in the livers of mice during chronic liver injury, proposing that the induction of senescence provides a barrier that limits liver fibrosis (Krizhanovsky et al., 2008). In the aforementioned study, the investigators showed that the lack of p53 results in excessive fibrotic tissue and increased expression of TGF-β, suggesting that the senescence program is p53-dependent. Thus, p53-deficient activated HSC can bypass the senescence response, continue to proliferate, and produce ECM within the tissue. It is noteworthy that the diverse factors secreted by senescent cells

attract various innate immune cells including macrophages, neutrophils, and NK cells that facilitate the final clearance of the senescent HSCs (Weiskirchen & Tacke, 2016).

3. Degradation of ECM

In order to complete the restoration process, and in addition to the loss of scar-producing myofibroblasts, degradation of the ECM components is a prerequisite for adequate fibrosis regression. This includes the activation of MMPs, the contribution of macrophages that phagocytize matrix fragments, as well as the reduction of MMP-inhibitory proteins (TIMPs) (Karsdal et al., 2017). During liver fibrosis, as mentioned previously, MMP activity is inhibited by TIMPs. However, there is fundamental evidence that during termination of fibrosis, there is a rapid reduction in TIMP levels mainly TIMP-1, tipping the overall MMP-TIMP balance, which results in increased matrix degradation activity and net degradation of scar tissue (Ramachandran & Iredale, 2012). The role of TIMP-1 in fibrogenesis and resolution was confirmed using transgenic systems, whereby hepatic TIMP-1 overexpression accelerated fibrogenesis, but also caused a failure of scar resolution (Yoshiji et al., 2002). Additionally, it has been shown that TIMP-1 has an antiapoptotic effect on hepatic myofibroblasts, demonstrating that the loss of TIMP-1 during recovery may also contribute to a decline in the myofibroblasts in the liver (Elsharkawy et al., 2005). This concept was proven in a study in which CCl_4-injected mice were treated with an MMP-9 mutant that acts as a scavenger for TIMP-1. This led to increased apoptosis of activated HSCs and reduced hydroxyproline content, a component of collagen, in the liver (Roderfeld et al., 2006). Overall, these data suggest that the

stimulation of MMP activity, or decrease of their inhibitors, should be therapeutically beneficial.

4. Shifting the Hepatic Microenvironment from Inflammation to Resolution

Being one of the most important factors during fibrosis, the inflammatory milieu in the liver usually shifts to a condition in which it is possible to rebuild the normal liver architecture after the arrest of chronic liver damage. In this resolution phase, the recuperating hepatocytes and their neighboring non-parenchymal cells emit restorative and anti-inflammatory signals. Consequently, major phenotypic adjustments of the immune cells, especially the stimulation of a restorative phenotype in macrophages and modulation of type 2 immunity, predominate in the liver (Gieseck, Wilson, & Wynn, 2018; Pakshir & Hinz, 2018). During this phase, one of the most striking phenotypic immune cell switches in mice is observed in macrophages that acquire a restorative phenotype that is characterized by $Ly6C^{low}$ expression and high expression of MMP-9 and MMP-12 (Ramachandran et al., 2012). Emerging evidence implicates macrophages as crucial mediators of fibrosis regression. Interestingly, macrophage depletion during the regression phase following chronic CCl_4 administration caused a failure to reduce the hepatic scar, an opposite outcome to that seen with depletion during fibrogenesis (Duffield et al., 2005). Likewise, C-C chemokine receptor 2 (CCR2) knockout mice exposed to chronic injury with CCl_4 had diminished macrophage recruitment and fibrogenesis, but also a hindered ability to resolve fibrosis (Mitchell et al., 2009). Also, the ability of macrophages to produce molecules such as TRAIL and MMP-9 promotes myofibroblast apoptosis, although studies

validating this mechanism are still lacking (Figure 5) (Ramachandran & Iredale, 2012). It is probable that all the divergent functional effects of macrophages are associated with the heterogeneity of these cells. Macrophages can adopt distinct functional characteristics depending on the stimuli to which they are exposed. Interestingly, a specific macrophage phenotype will predominate during fibrosis regression, which may be distinct from the phenotype that promotes fibrogenesis (Figure 4 and 5). Determining if the same macrophage population switches from a profibrotic to pro-resolution phenotype *in situ,* and identifying the factors mediating this switch, may enable the development of novel therapies designed to promote this change *in vivo* and thus induce fibrosis resolution.

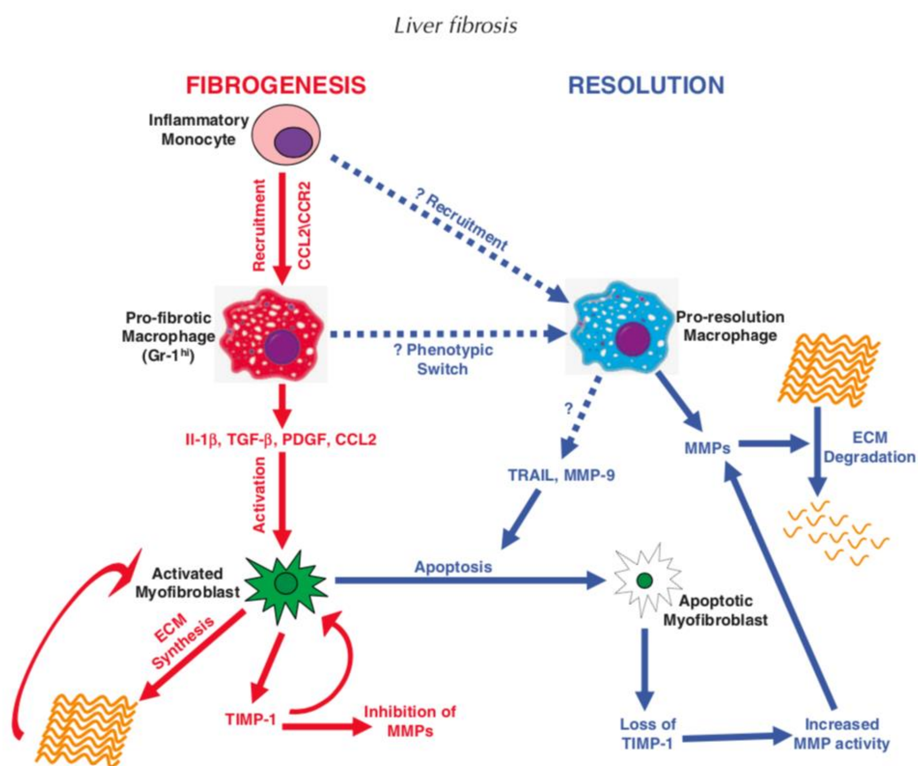


Figure 5. **Macrophages as crucial players of liver fibrogenesis and fibrosis resolution** adapted from (Ramachandran & Iredale, 2012).

In addition to the role of macrophages, the restorative milieu within the liver is enriched with other immune cells including DCs, NK and T cells. In an elegant study, Jiao et al., demonstrated that fibrosis regression is delayed in CCl_4-injected mice in which DCs were conditionally depleted (Jiao et al., 2012). The authors showed that artificial administration of DCs either by adoptive transfer of purified DCs or FMS-like kinases-3 ligand (FLT3L), accelerated fibrosis regression. Additionally, studies have determined that NK cells induce apoptosis of activated HSCs *via* the transmembrane protein NKG2D and the cytokine TRAIL (Tian, Chen, & Gao, 2013), and that T cells limit hepatic inflammation and fibrosis by co-localizing with HSCs and stimulating its apoptosis by a cell-cell contact-dependent manner (Hammerich et al., 2014).

The multiplicity of molecular and cellular triggers involved in initiation, progression and resolution of hepatic fibrogenesis offers numerous therapeutic possibilities. Thus, the understanding of the biology of fibrosis resolution is likely to inform novel treatment options.

E. Statins and Liver Injury

Statins have a major role in reducing cholesterol levels by inhibiting the rate limiting enzyme, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonate (Lennernas & Fager, 1997). The liver is the major site of cholesterol biosynthesis, and the inhibition of this central pathway by statins up-

regulates hepatic low-density lipoprotein (LDL) receptors and lowers proatherogenic circulating LDL cholesterol (Figure 6) (Argo, Loria, Caldwell, & Lonardo, 2008).

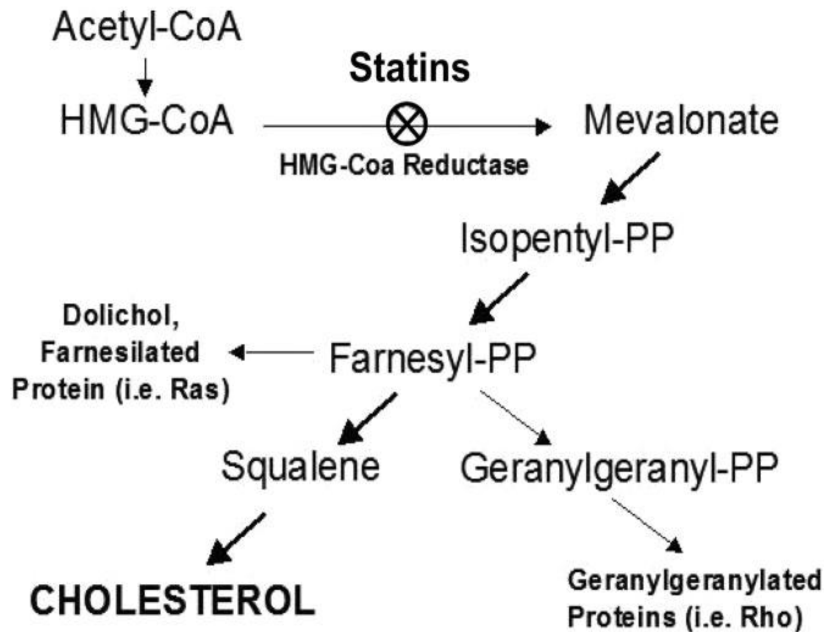


Figure 6. **Mechanism of action of statins** adapted from (Argo et al., 2008).

Beyond their cholesterol-lowering properties, statins are widely known for their pleiotropic effects including their anti-inflammatory, antioxidant and antiproliferative capacities (Jasinska, Owczarek, & Orszulak-Michalak, 2007). Many of these pleiotropic effects are mediated through the inhibition of isoprenoid formation, which is important for farnesylation or geranylgeranylation of proteins involved in gene synthesis and cell regulation (S. Sebti & Hamilton, 1997; S. M. Sebti, 2005). This inhibition subsequently

decreases the activity of the Rho-GTPase family, especially RhoA and its downstream effector Rho-kinase (ROCK) (Rikitake & Liao, 2005; Trebicka et al., 2007).

Recently, several studies have shown that statins might offer clinical benefits in the setting of liver diseases that include NAFLD, cholestatic liver disease, and cirrhosis (Pastori et al., 2015; Schierwagen et al., 2016; Tsochatzis & Bosch, 2017). Park et al. have revealed the hepatoprotection effect of statins in MCD-induced NASH and showed that its effect is mediated by activating peroxisome proliferator-activated receptor alpha (PPARα) and increasing hepatic mitochondrial and peroxisomal Fatty Acid Oxidation (FAO) (H. S. Park et al., 2016). Likewise, several studies have focused on reducing intrahepatic resistance and portal pressure in cirrhotic rats to avoid vasoconstriction. In this context, it was shown that statins inhibit the RhoA/Rho-kinase pathway, and increase the activity of endothelial nitric oxide synthase (eNOS) and NO availability in cirrhotic rats (Trebicka et al., 2007; Trebicka & Schierwagen, 2015). Moreover, a novel RhoA-downstream effector termed Kruppel-Like Factor 2 (KLF2) was shown to be a vasoprotective transcription factor (Gracia-Sancho et al., 2011). Subsequently, by inhibiting RhoA by statins, KLF2 expression is up-regulated, leading to reduced endothelial dysfunction and decreasing portal hypertension (Marrone et al., 2015; Marrone et al., 2013; Trebicka & Schierwagen, 2015). Statins also exhibit antifibrogenic properties through the inhibition of proliferation and induction of senescence in hepatic myofibroblasts in bile duct-ligated rats (Klein et al., 2012; Trebicka et al., 2010). The possible intracellular mechanisms of statins in liver cells are shown in Figure 7 (Trebicka & Schierwagen, 2015).

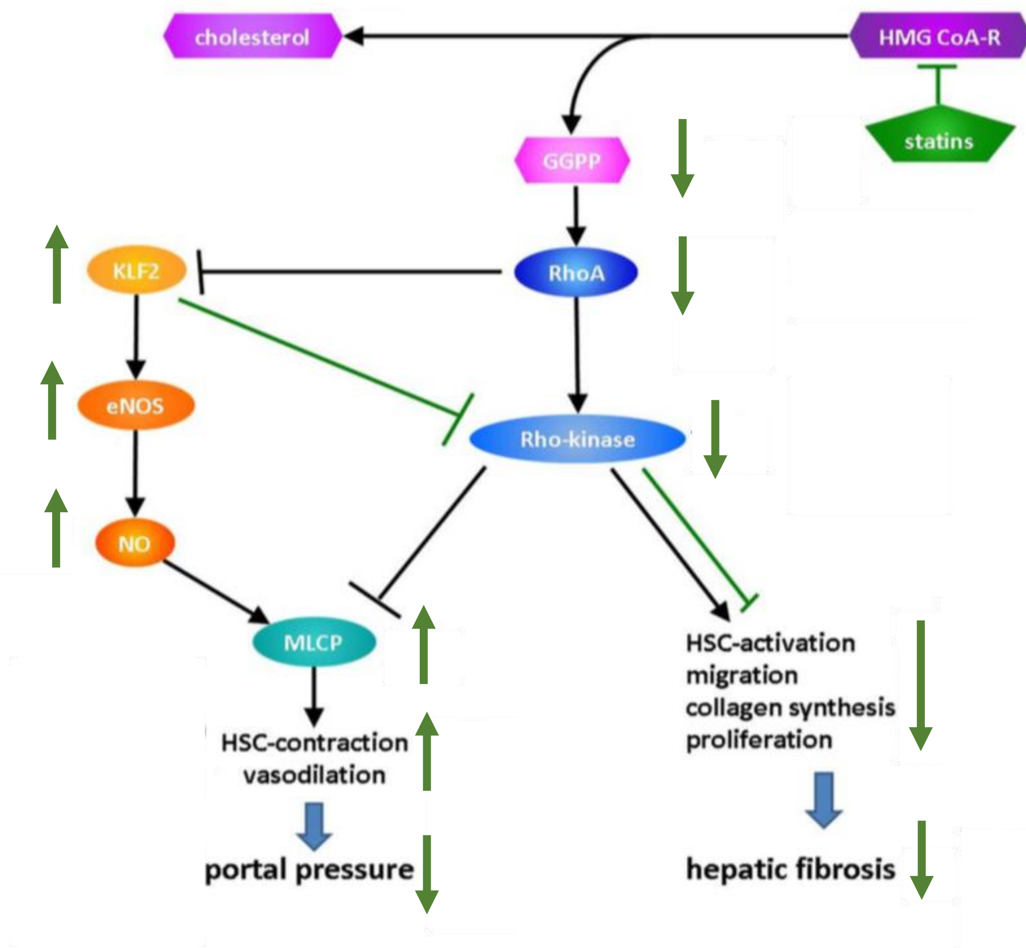


Figure 7. **Schematic overview of mechanism by which statins (in green) diminishes liver fibrosis and reduce portal pressure** adapted from (Trebicka & Schierwagen, 2015).

Therefore, these collective effects that promote the healing of liver diseases require further investigation so that they may be potentially useful in the management of inflammation, enhancement of liver regeneration, and reduction of oxidative stress.

CHAPTER II

AIM OF THE PROJECT

Previous studies have demonstrated the antiproliferative and apoptotic effect of statins on isolated human hepatic myofibroblasts (Mouawad et al., 2016). Additionally, our preliminary data showed that pitavastatin exerts an anti-inflammatory effect on macrophages *in vitro*. The objective of our study is to investigate the effect of pitavastatin on the molecular mechanism of hepatic fibrosis.

Specific Aims:

1. To establish the CCl_4-induced liver fibrosis model in our lab.
2. To investigate whether pitavastatin has an antifibrotic effect, and to elucidate the mechanisms underlying this effect.
3. To determine whether pitavastatin will accelerate the regression of liver fibrosis, and if it does, to investigate the underlying inflammatory effect.

CHAPTER III

PREVIOUS STUDIES AND PRELIMINARY DATA

A. Previous studies

Previous studies performed by our group have suggested that statins inhibit (1) inflammation in isolated monocytes and (2) the proliferation of the hepatic myofibroblasts in culture.

*1. **Modulation of COX-2 Expression by Statins in Human Monocytic Cells** (Habib et al., 2007)*

In this study, simvastatin and mevastatin decreased the proinflammatory cyclooxygenase-2 (COX-2) and its metabolite the prostaglandin E_2 (PGE_2) in the human monocytic cell line U937 in response to LPS as shown in Figure 8 adapted from (Habib et al., 2007)

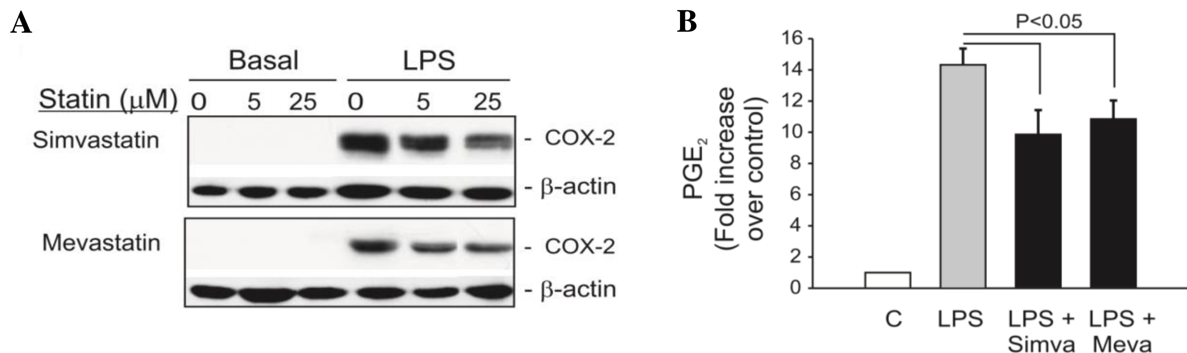


Figure 8. **Effect of simvastatin and mevastatin on LPS-induced human monocytes** adapted from (Habib et al., 2007). U937 was differentiated using PMA and incubated in the presence or absence of 5 μg/ml LPS and 5 or 25 μM simvastatin or mevastatin for 24 hours. (A) Western blot analysis of COX-2 and β-actin. (B) PGE_2 synthesis was measured in the supernatant of the cells using enzyme immunoassay. Results are expressed as Mean ± SEM (n=8) (paired t-test).

2. ***Statins Modulate Cyclooxygenase-2 and Microsomal Prostaglandin E Synthase-1 in Human Hepatic Myofibroblasts*** *(Mouawad et al., 2016)*

In this study, our group demonstrated that simvastatin and fluvastatin blocked the proliferation of human hepatic myofibroblasts in culture through an increase in cAMP and an induction of COX-2/mPGES-1 pathway (Figure 9).

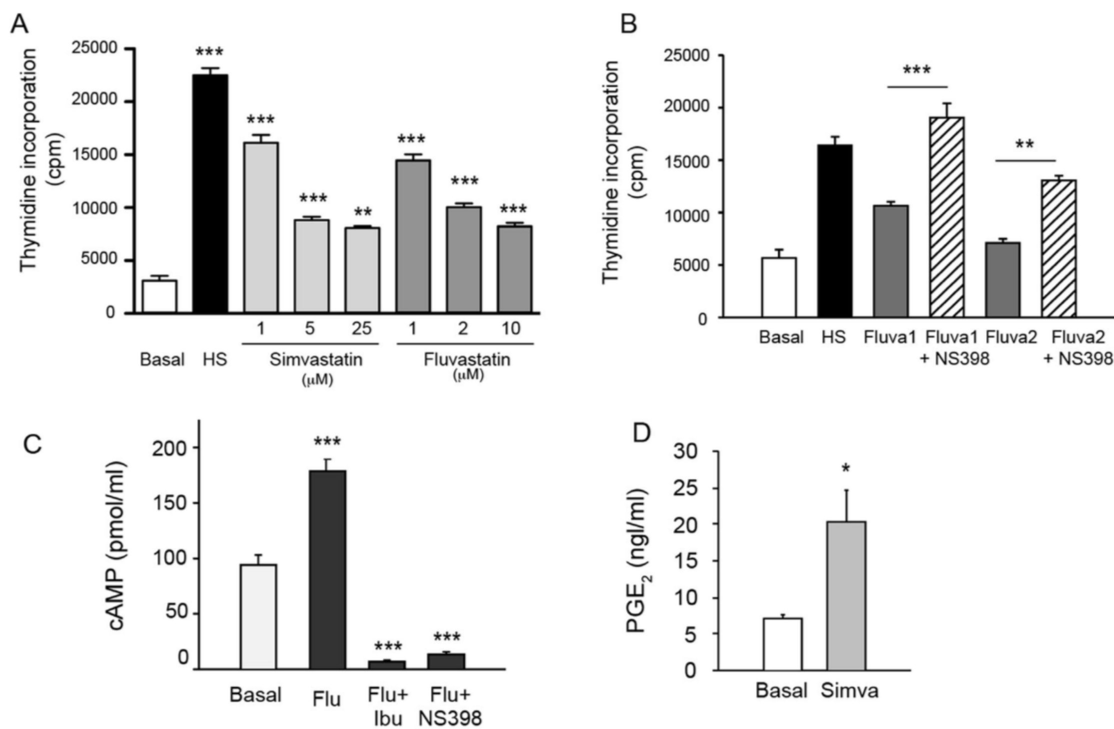


Figure 9. **Effect of statins on DNA synthesis and release of PGE_2 in human myofibroblasts** adapted from (Mouawad et al., 2016). (A) Human myofibroblasts were activated by 5% normal human serum (HS) alone or in the presence of different concentrations of statins for 24 hours. One microcurie of [^{3}H] thymidine was added per well during the last 6 hours of incubation. Data are expressed as Mean ± SEM (n=3) (one-way ANOVA followed by Dunnett test). (B) cells were pretreated with 5 µM NS398 for 15 min prior to the addition of 1 or 2 µM Fluvastatin and HS for 24 hours. (C) Effect of COX-2 inhibition on statin-induced cAMP release. Cells were treated with 50 µM Ibuprofen or 5 µM NS398 for 15 min prior to incubation with 10 µM Fluvastatin for 10 min. (D) PGE_2 was assessed in the supernatant of cells treated in serum-free medium with 25 µM of simvastatin for 48 hours. Data are expressed as Mean ± SEM (n=3) (unpaired t-test).

B. Preliminary Data: Effect of Statins on Bone-Marrow Derived Macrophages

Recently, in the group of Dr. Habib, we evaluated the effect of newly developed statins on the LPS-dependent induction of inflammation of macrophages. I participated in the assessment of the effect of two statins, pitavastatin and atorvastatin, on the IL-6 and TNF-α secretion levels in bone-marrow derived macrophages (BMDMs) in response to LPS. As shown in Figure 10, BMDMs treated with 10 ng/ml LPS showed a significant increase in the secretion of both IL-6 and TNF-α cytokines compared to unstimulated cells (control). When BMDMs were pretreated with 10 µM pitavastatin 15 min prior to the addition of LPS, a significant decrease in both IL-6 (34%) and TNF-α (45%) was obtained.

All these results obtained *in vitro* on cultured macrophages and human myofibroblasts prompted us to test the effect of pitavastatin rather than atorvastatin, and to evaluate its effect *in vivo* in an animal model of chronic liver fibrosis where inflammation and macrophages are known to play a role in initiating and maintaining liver fibrosis.

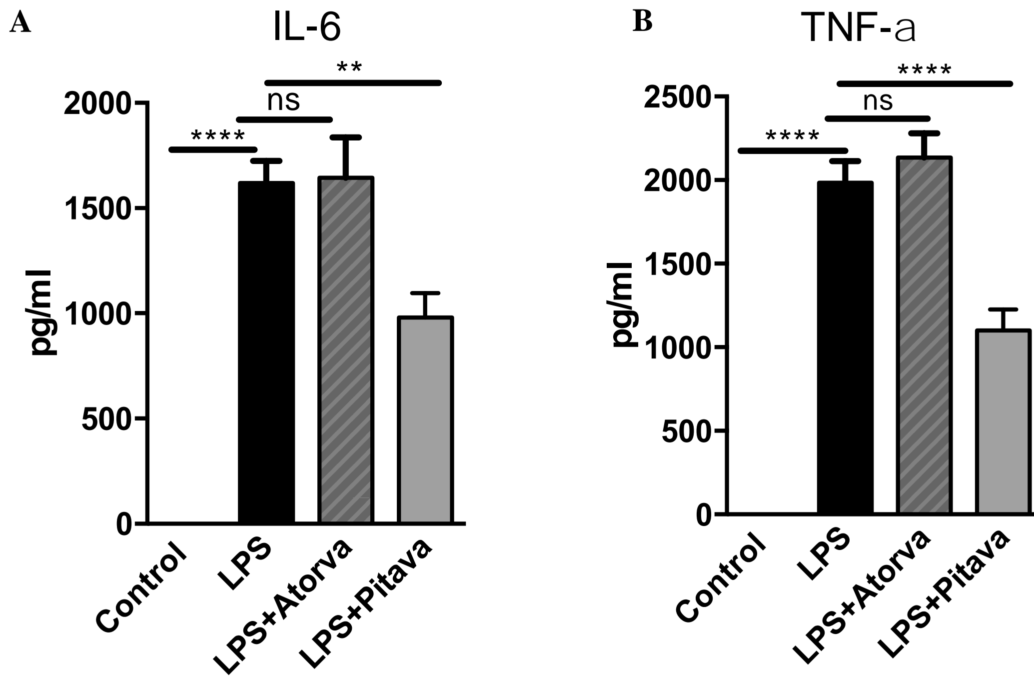


Figure 10. **Effect of statins on LPS-induced IL-6 and TNF-α formation in BMDMs.** BMDMs were pretreated with 10 µM of atorvastatin (Atorva) or pitavastatin (Pitava) 15 min prior to the addition of 10 ng/ml of LPS for 6 hours. Cytokine levels were measured by ELISA. (A) IL-6 and (B) TNF-α levels upon Atorva and Pitava treatment. Data are expressed as Mean ± SEM (n=3). $^{**}P < 0.005$, $^{****}P < 0.0001$, ns not significant (one-way ANOVA followed by Tukey's test).

CHAPTER IV

MATERIALS AND METHODS

A. Animals Experiments

C57BL6/J mice were housed and fed at libitum at the Animal Facility of the American University of Beirut. All *in vivo* experiments were performed with male mice at 10-11 weeks of age under ethical conditions approved by the Institutional Animal Care and Use Committee (IACUC) at American University of Beirut (IACUC Approval# 17-07-421).

B. Liver Injury Models

1. To Study AntiFibrotic Effect

For antifibrotic studies, chronic liver injury was induced by injecting carbon tetrachloride (CCl_4) (Sigma, Cat# 270652), solved in mineral oil (MO) (Sigma, Cat# M5310), i.p. at 0.6 ml/kg body weight two times per week for 4 weeks. Pitavastatin (Cayman Chemical Company, Ann Arbor, MI, USA Cat# 15414) was solved in dimethyl sulfoxide (DMSO) (Amresco, Cat# 67685). Pitavastatin, or equal amount of vehicle (DMSO, Saline; 1:25; v/v), was administrated daily i.p. at 10 mg/kg body weight starting the first day of the third week.

2. To Study Regression Effect

Chronic liver injury was induced by injecting CCl_4, solved in MO, i.p. at 0.6 ml/kg body weight two times per week for 6 weeks. Pitavastatin, or an equal amount of vehicle as previously described, was administered i.p. at 10 mg/kg body weight 2 hours prior to the last injection of CCl_4 and daily until the sacrifice. Mice were sacrificed 24 and 96 hours after last CCl_4 injection for final analysis.

C. Serum ALT/AST

Whole blood was collected using the retro-orbital puncture technique. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured at the Plateforme de Biochimie INSERM U1149, Paris, France.

D. Histological and Immunohistochemical Examination

1. Sirius Red Stain

Collagen fibers were detected after Sirius Red staining (Polysciences, Inc. Warrington, PA, USA Cat# 24901-250). Liver tissues were fixed in 10% formalin, embedded in paraffin, and then sliced into serial sections of 4 µm thickness. Staining was performed according to standard protocols (Mr. Nabih Mheidly, Histology Lab, Department of Anatomy, Cell Biology, and Physiological Sciences, American University of Beirut).

2. *Immunohistochemistry*

Immunohistochemistry using a mouse monoclonal antibody for α-SMA (Sigma Aldrich, St. Louis. MO, USA, Cat# A2547) was performed in paraffin-embedded sections as previously described (Teixeira-Clerc et al., 2006). After blocking for endogenous hydrogen peroxide, a biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Inc. Cat# BA-9200) was used. Finally, sections were counterstained with hematoxylin (Leica Biosystem, Ref# RE7107).

E. RNA Extraction, Reverse Transcription and Real-time Polymerase Chain Reaction

1. *RNA Extraction*

Total RNA was extracted from the liver using 1ml QIAsol (QIAGEN, Cat# 79306). After homogenizing the liver using the Tissue Lyser (QIAGEN II), total RNA was extracted using qiazol and MN mini RNA Plus kit according to (Lodder et al., 2015)and resuspended into 60 µL of RNase, DNase free water and quantified using a Spectrophotometer Nanodrop (Thermo Fisher Scientific).

2. *Reverse Transcription (RT)*

Reverse transcriptase reaction was performed using 2 µg of total RNA in a final volume of 20 µL according to the manufacturer's instructions (Thermo Fischer Scientifics, Cat# 00407363), and the RT-PCR apparatus (Bio-Rad Laboratories, California, USA) as follows: 10 min at 25°C, 2 hours at 37°C followed by 5 min at 85°C and ends at 4°C. The cDNA samples were stored at -20°C.

3. Real-Time PCR

Real-time PCR reactions were performed using the CFX384 system (Bio-Rad Laboratories, California, USA) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, California, USA) as follows: one cycle at 94°C for 15 minutes, 50 cycles at 94°C for 15 seconds, 56°C for 9 seconds each, and finally one cycle at 72°C for 30 minutes. Melting curves were evaluated to check for primer specificity for the PCR product and the results were quantified and analyzed using the Delta-Delta CT method. The primer sequences used were according to (Lodder et al., 2015). The housekeeping gene 18S was used for normalization. Mouse PDGFRβ primers were obtained from Bio-Rad (Cat# 10025636).

F. Extraction of Protein

Total protein was extracted from liver using 500 µl protein lysis buffer (50 mM HEPES pH 7, containing: 1% Nonidet P40, 2% glycerol, 137 mM NaCl, 1 mM EDTA pH 8, 10 mM sodium floride (NaF), 1 mM othrovanadate, 40 mM beta-gylcerophosphate, 0.1mM dithiothreitol (DTT), and water). After homogenizing the liver using the Tissue Lyser (QIAGEN II), total protein was extracted according to (Hegde et al., 2018) and quantified using DC compatible protein assay kit (Bio-Rad, Cat# 500-0112) with bovine serum albumin (BSA) as standard.

G. ELISA

ELISA assays (IL-6, Thermo Fischer Scientifics, Cat# 88-7064-22) was performed on 200 µg protein stored at -20˚C. Assays were performed according to the manufacturer

instructions. Plates were read via spectrophotometer using the ELISA Plate reader at λ=450nm.

H. Hepatic leukocytes isolation

Briefly, mouse livers *in situ* were perfused with 10 ml phosphate-buffered saline (PBS) solution through the portal vein. Livers were then harvested and digested in Hank's balanced salt solution (HBSS) containing Liberase™ (Roche Diagnostics, Cat#291963) for 30 min at 37 °C. Digested livers were passed through 100-μm cell strainers and contaminating hepatocytes were removed by centrifugation at 60g for 2 min. Hepatic leukocytes were separated by Ficoll-Paque™ Plus (GE Healthcare Bioscience AB, Uppsala, Sweden Cat# 17-1440-03) and stained using fluorescence-activated cell sorting (FACS) staining.

I. Flow Cytometry

Multicolor fluorescence-activated cell sorting (FACS) staining was conducted using combinations of the following monoclonal antibodies: CD45; TCRβ; CD19; Ly6G; CD11B; F4/80; and Ly6C as described in (Hegde et al., 2018). Dead cells were excluded by Live/Dead Fixable Blue Dead Cell Stain, for UV excitation. The analysis was performed using FACS Aria-II-SORP cell sorter (BD Biosciences). Data were analyzed with FlowJo Virgin 10, LLC software.

J. Statistical Analysis

All experimental data are presented as mean ± standard mean of error (SEM). Differences between groups were evaluated by Mann-Whitney U test. A value of $P < 0.05$ was considered statistically significant (GraphPad Prism 6, GraphPad Software Inc., La Jolla, CA 92037, USA).

CHAPTER V

RESULTS

A. Establishing a Model of Chronic Liver Injury in Mice

1. Experimental Design

We first aimed to establish the CCl_4-induced chronic injury model in the lab by CCl_4 intoxication in 10-11 week old C56BL/6 males by i.p. injections of 0.6 ml/kg CCl_4, solved in MO (1 :10), twice a week for 6 weeks. 5 mice received a vehicle of mineral oil only. 24 hours after the last injection of CCl_4, the control mice and 11 CCl_4-injected mice were sacrificed, and another 11 mice were sacrificed four days after the last injection of CCl_4 (Figure 11). From these mice, we obtained blood and liver sections for the purpose of molecular and histological analysis. All sacrificial procedures were performed according to ethical standards.

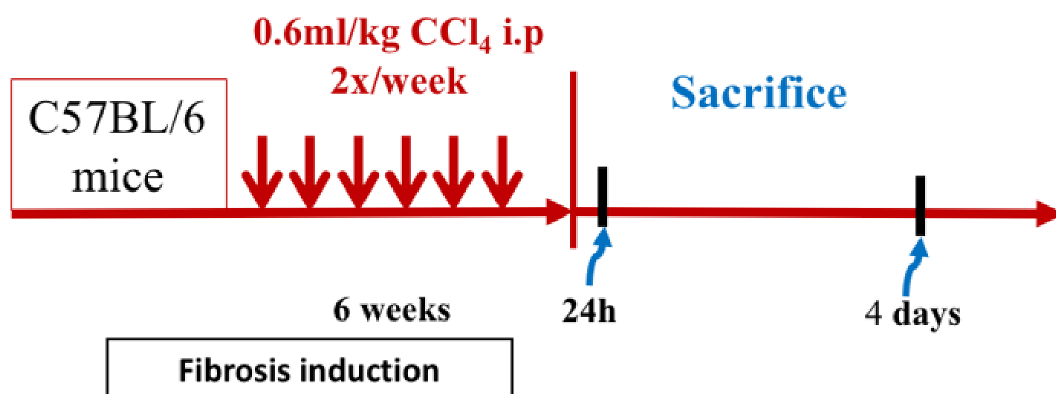


Figure 11. **Repeated injections of CCl_4 induce chronic liver fibrosis in mice.** C57BL/6 mice were given i.p. injections of CCl_4 twice a week for 6 weeks and analyzed during regression without additional treatment at the indicated time points.

2. Assessment of Chronic Liver Injury.

Hepatic Injury was first assessed by measuring the levels of aminotransferase activity in the serum (Figure 12). An increase in alanine and aspartate aminotransferase levels was evident when compared to MO at 24 hours after the last injection of the repetitive CCl_4 injections. These increases were approximately 1000 and 10000-fold, for ALT and AST, respectively.

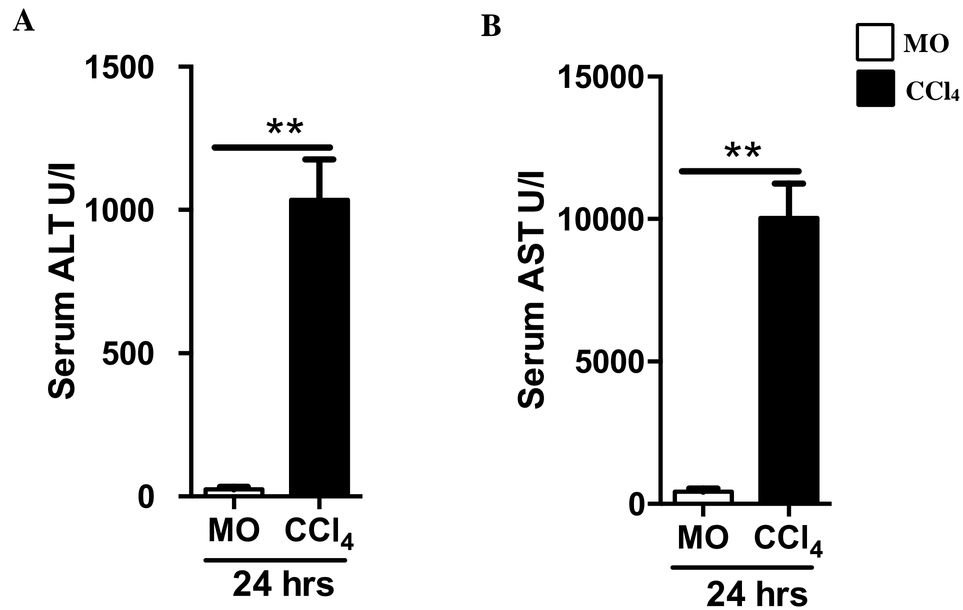


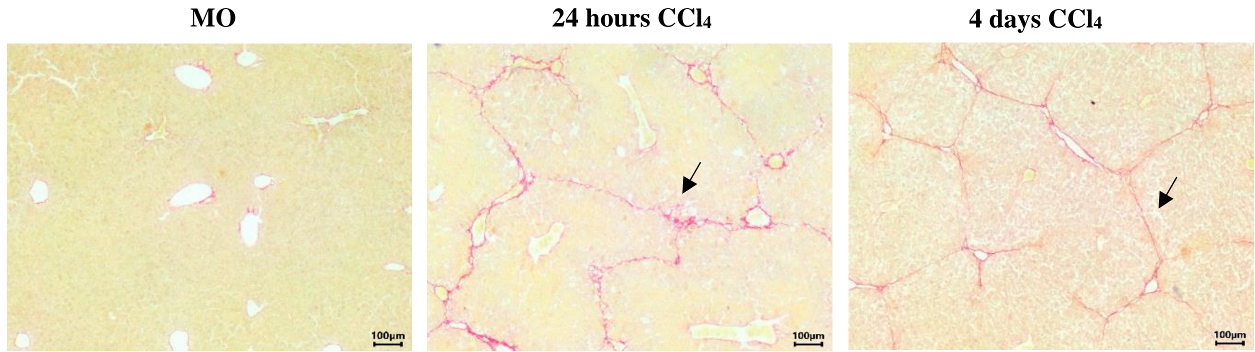
Figure 12. **CCl_4 induces hepatic injury.** Hepatic injury was assessed by (A) ALT and (B) AST levels. Data are expressed as mean ± SEM (n=5 for MO, 11 for CCl_4); $*P < 0.05$, (Mann-Whitney U test).

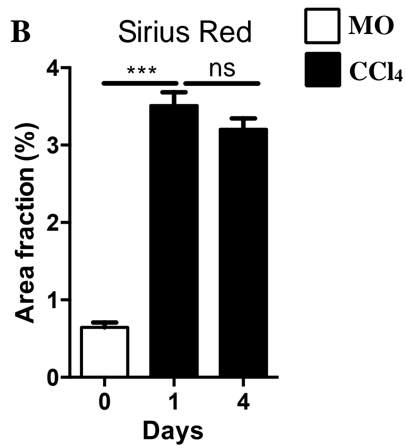
3. Histological Assessment of Liver Fibrosis.

a. Collagen

Previous studies have shown that liver fibrosis is associated with high deposition of collagen of type I and III. In order to examine the effect of CCl_4 on collagen deposition in our model, Sirius Red (SR) staining was performed, which stains collagen fibers in red. Figure 13A shows an increase in the SR stains, indicating a deposition of collagen in CCl_4-treated mice compared to MO mice at the 24 hours and 4 days post–last CCl_4 injection. We used the ImageJ software to assess the percentage of the stained area from the acquired images. Collagen deposition in the liver was significantly higher in the group of mice sacrificed 24 hours after the last injection of CCl_4 compared to the mineral oil control group (Area fraction 3.5% and 0.6 %, respectively) (Figures 13B and C). However, there was no difference in the percentages of collagen between the groups of mice sacrificed 24 hours and 4 days after the last injection of CCl_4, suggesting that the fibrosis was maintained at 1 and 4 days after cessation of CCl_4 injections as shown previously by (Ramachandran et al., 2012)

A





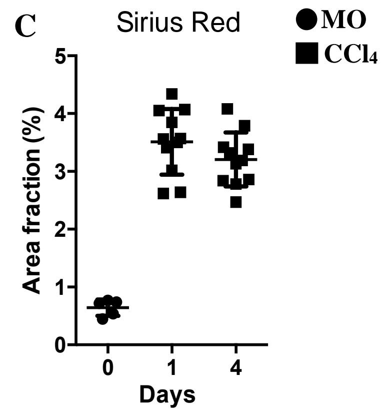


Figure 13: **Sirius Red staining of collagen fibers during liver injury**. (A) Representative images of Sirius red staining, indicated with an arrow, performed on paraffin sections. Scale bar = 100µm. (B) Quantification of stained area using ImageJ software. Data represented as (B) Histogram and (C) Side scatter plot. Data are expressed as mean ± SEM (n=5 for MO, 11 for CCl_4). ***$P < 0.001$; ns not significant (Mann-Whitney U test).

b. alpha- Smooth Muscle Actin (α-SMA).

During liver fibrosis, activated myofibroblasts generate large quantities of α-SMA. We thus performed immunohistochemistry of α-SMA on de-paraffinized tissue sections from CCl_4-treated mice versus control mice using the selective antibody anti α-SMA. Figure 14 showed an increase in the expression of α-SMA proteins by myofibroblasts 4 days after cessation of CCl_4. The percentage of area fraction, determined by ImageJ software, indicated a significant increase in α-SMA in CCl_4-treated mice (3.0 % of Area Fraction) compared to the control mice (0.4 %) at the indicated time point (Figure 14).

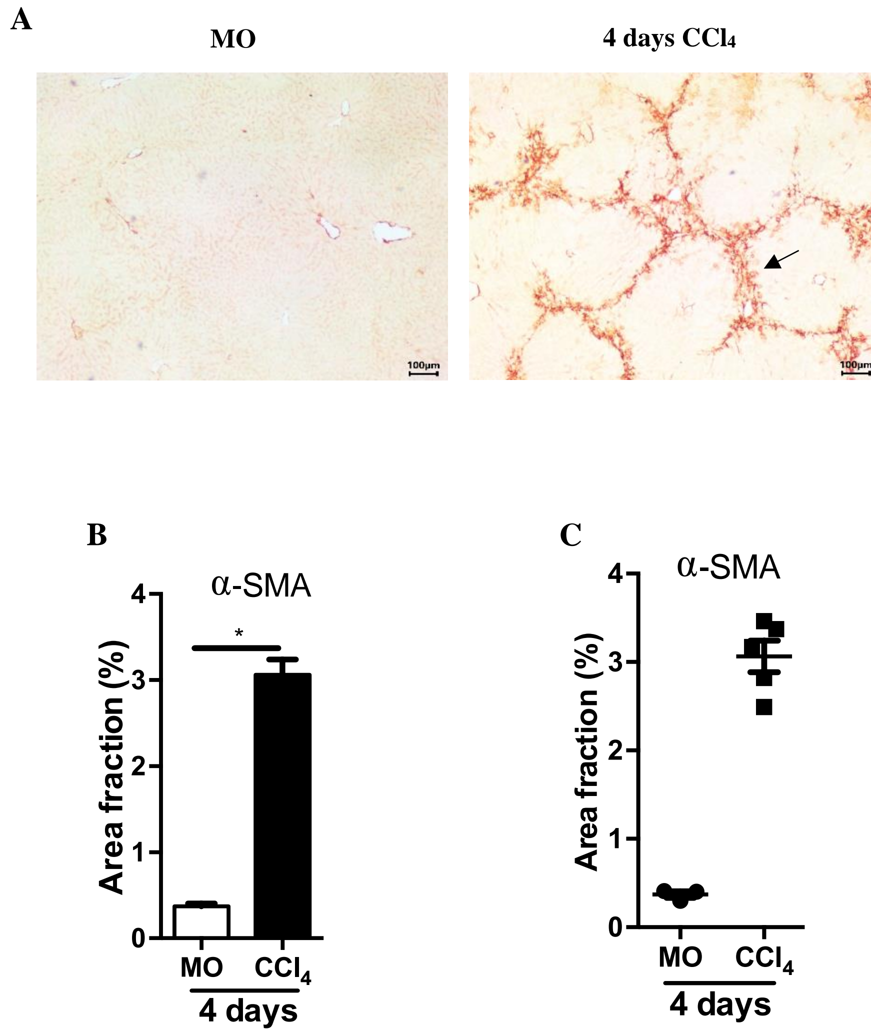


Figure 14: **Immunohistochemistry of α-SMA on liver sections**. (A) Representative images of liver sections from (a) control MO mice or (b) CCl_4-injected mice 4 days post last injection and α-SMA protein indicated with an arrow. Scale bar =100µm. (B-C) α-SMA expression quantified using ImageJ software. Data are expressed as mean ± SEM (n=3 for MO, 5 for CCl_4). $^{*}P < 0.05$ (Mann-Whitney U test).

4. Gene Expression.

a. <u>Effect of CCl_4 on the Expression of Fibrotic and TIMP-1 genes.</u>

The previous findings were corroborated by gene expression analysis of the fibrotic genes α-SMA, TGF-β and CTGF. Hepatic mRNA levels of these genes were increased in mice sacrificed 24 hours after the last injection of CCl_4 compared to the MO group. However, when assessing the expression of the fibrotic genes after 4 days of CCl_4 cessation, a decrease in the mRNA levels of the α-SMA and CTGF, but not the TGF-β genes, was detected compared to the day 1 group (Figure 15A). Similar results were obtained for TIMP-1 expression (Figure 15B).

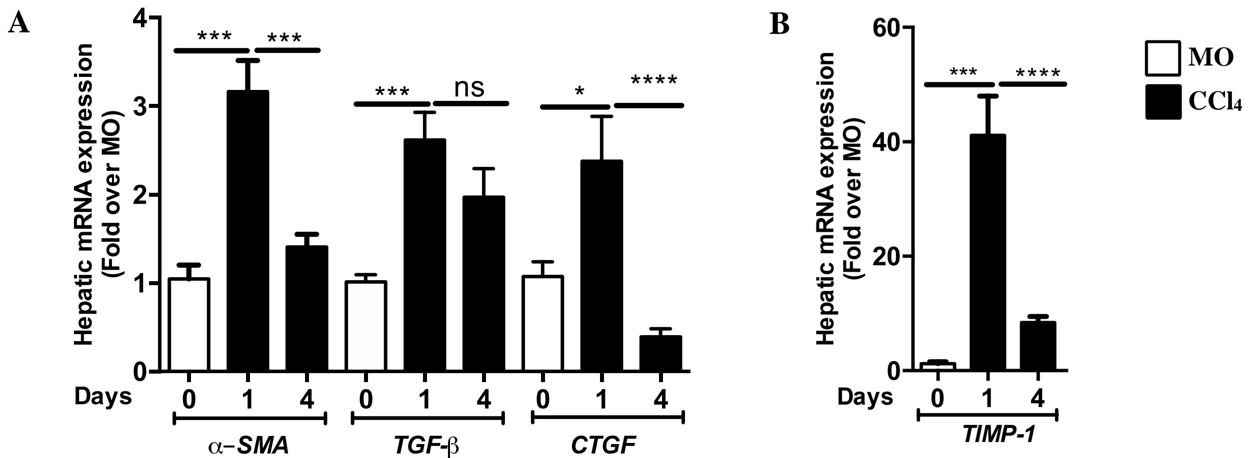


Figure 15: **Gene expression of fibrotic genes**. RT-PCR was performed on RNA samples from control and CCl_4-treated mice at 24 hours and 4 days after CCl_4 cessation. α-SMA, TGF-β and CTGF gene expressions were elevated at the 24 hours timepoint. Moreover, α-SMA, CTGF and TGF-β gene expressions decreased at the 4 days timepoint, albeit not a significant decrease for the latter. 18S mRNA expression was used for normalization. Data are expressed as mean ± SEM (n=5 for MO, 11 for CCl_4). **P* < 0.05; ****P* < 0.001; *****P* <0.0001; ns not significant (Mann-Whitney U test).

b. Effect of CCl_4 on the Expression of Inflammatory Genes.

Hepatic mRNA expression of CCL2, CCL3, and CCL4 genes was assessed for the mice sacrificed 24 hours after the last injection of CCl_4 and compared to the MO group. Gene expression analysis showed elevated levels of all the aforementioned genes, corresponding to around 71-fold for CCL2, 37-fold for CCL3, and 93-fold for CCL4 (Figure 16).

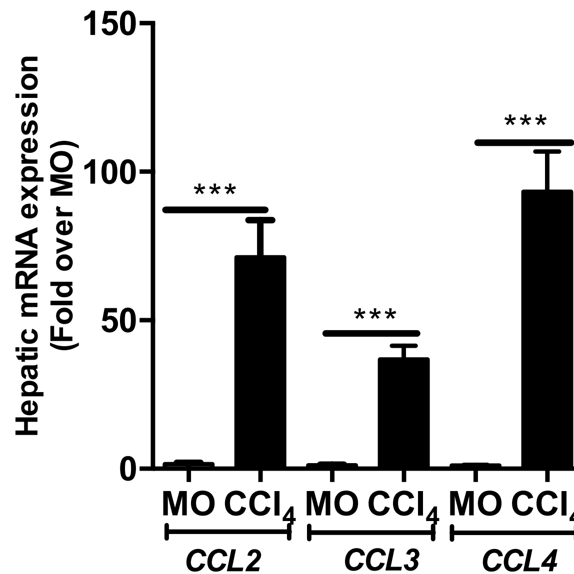


Figure 16. **Gene expression analysis of inflammatory genes**. RT-PCR was performed on RNA samples from control and treated mice with CCl_4 at 24 hours for CCL2, CCL3 and CCL4 genes. mRNA levels were elevated at the 24 hours timepoint. 18S mRNA expression was used for normalization. Data are expressed as mean ± SEM (n=5 for MO, 11 for CCl_4). ****P* < 0.001 (Mann-Whitney U test).

B. Antifibrotic Effect of Pitavastatin on Liver Fibrosis

1. Experimental Design

The pleiotropic effects of statins have long been documented, among which is their anti-inflammatory role. To assess whether the most nascent statin, pitavastatin, has an antifibrotic effect in the liver, all mice were first given injections (i.p.) of 0.6 ml/kg CCl_4 twice per week for 4 weeks. At the start of the third week, 16 mice were given daily injections of 10 mg/kg pitavastatin for 2 weeks along with the CCl_4 injections, and 16 mice were given injections of equivalent amount of DMSO (1:25) as a vehicle. Additionally, 3 mice were used as controls and were given injections of MO and vehicle (Figure 17). The 32 mice were sacrificed at 2 timepoints; 24 hours after the last injection of CCl_4 (3 MO-treated mice, 8 mice treated with CCl_4 + vehicle, and 6 mice treated with CCl_4 + pitavastatin) and at day 3 post CCl_4 (8 mice treated with CCl_4 and vehicle, and 10 mice treated with CCl_4 and pitavastatin).

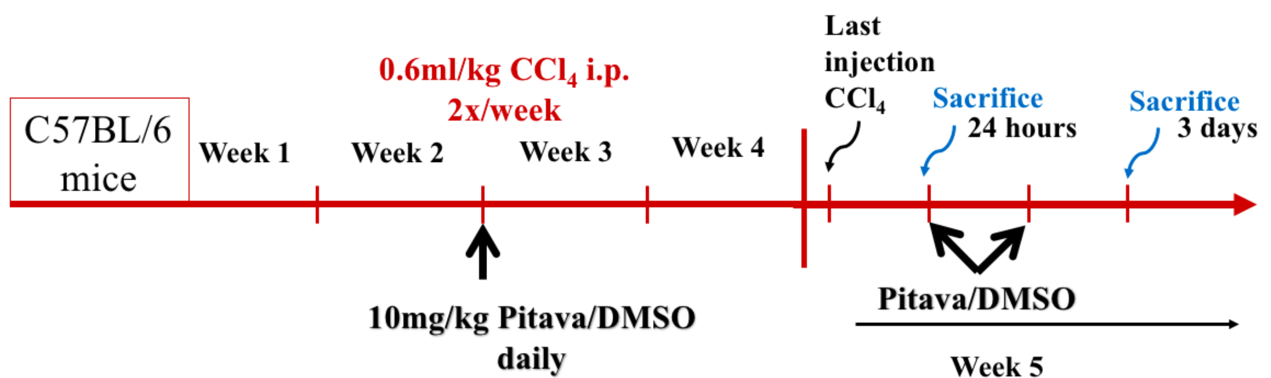


Figure 17**: Antifibrotic effect of pitavastatin on liver fibrosis**. C57BL/6 mice were treated with CCl_4 twice a week for 4 weeks. At the start of the third week, mice were given 10 mg/kg (i.p.) pitavastatin, or an equivalent amount of DMSO, every day until their sacrifice.

2. *Effect of Pitavastatin on Liver Aminotransferases*

We first checked the effect of pitavastatin on hepatic injury for the 24 hours group by assessing the level of aminotransferase activity in the serum (Figure 18). An increase in alanine and aspartate aminotransferase levels, 904 ± 58 and 6291 ± 635 U/I respectively, was evident in the CCl_4-injected group compared to the MO. However, pitavastatin treatment did not show any effect on the level of the hepatic enzymes when compared to the CCl_4-treated mice.

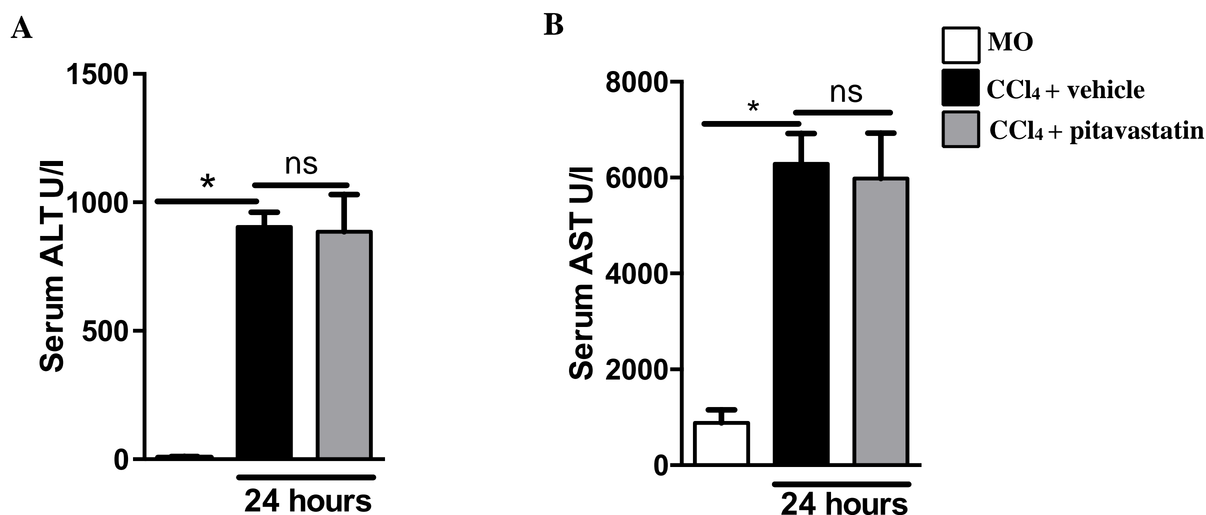


Figure 18. **Effect of pitavastatin on serum aminotransferases**. Hepatic injury was assessed by (A) serum ALT and (B) serum AST levels at 24 hours after the last CCl_4 injection in MO, CCl_4+vehicle and CCl_4+pitavastatin. Data are expressed as mean ± SEM (n=8 for CCl_4+vehicle, 6 for CCl_4+pitavastatin and 3 for MO). *$P < 0.05$; ns not significant (Mann-Whitney U test).

3. Pitavastatin Reduces Deposition of Collagen in the Injured Liver

We then examined the synthesis and deposition of ECM proteins by assessing, specifically, the modulation of the levels of collagens type I and III in the liver. Liver sections were stained with Sirius Red. Results showed an increase in the deposition of collagen in CCl_4-treated mice compared to control mice at 24 hours and day 3 groups. Representative images are shown (Figure 19A), and the percentage (area fraction) of collagen deposition was analyzed using ImageJ software (Figure B and C). For the mice sacrificed 24 hours after the last injection of CCl_4, the administration of pitavastatin resulted in a 33% decrease in Sirius Red staining when compared to the CCl_4 mice. A similar decrease was obtained for the day 3 group.

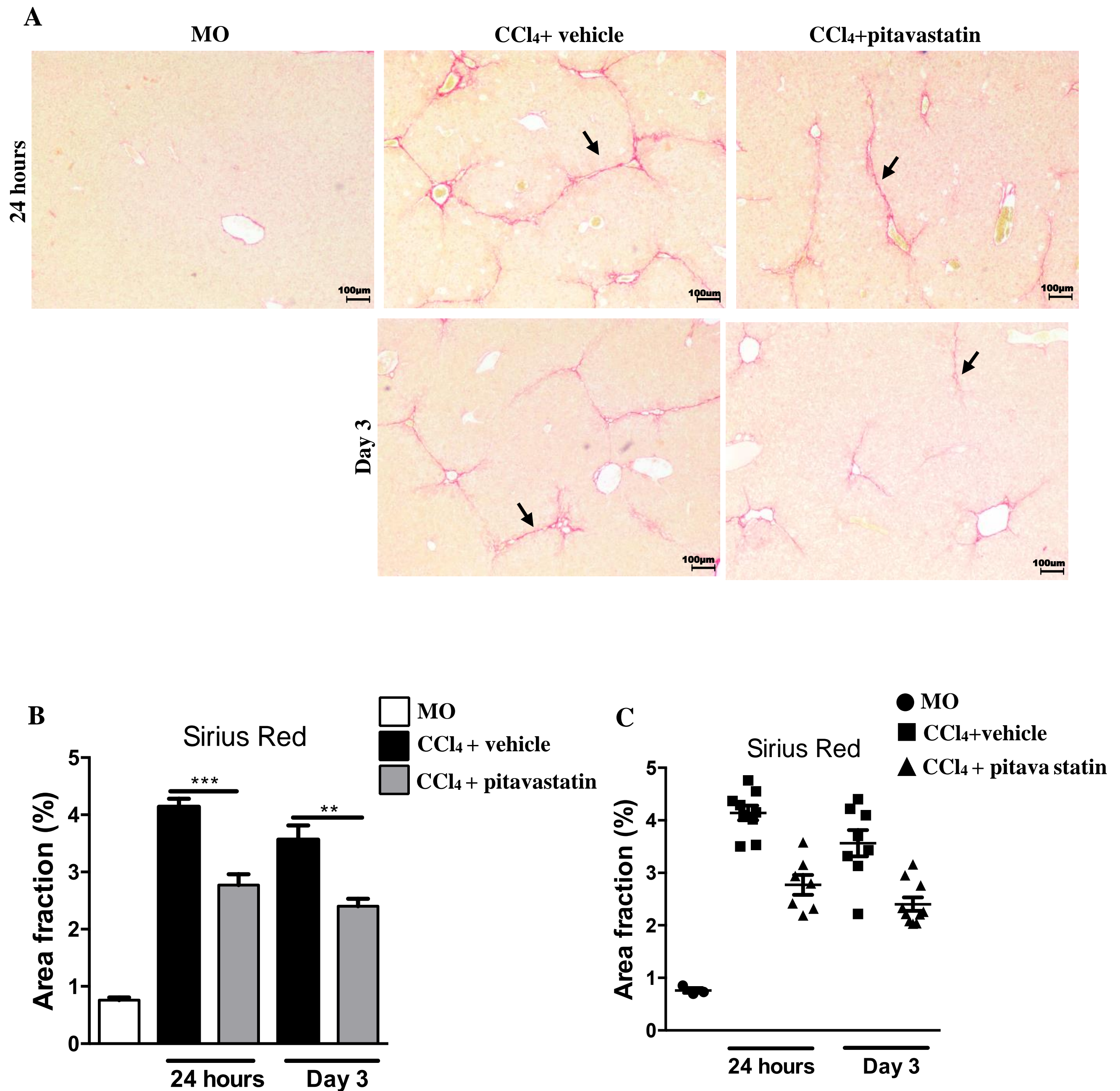
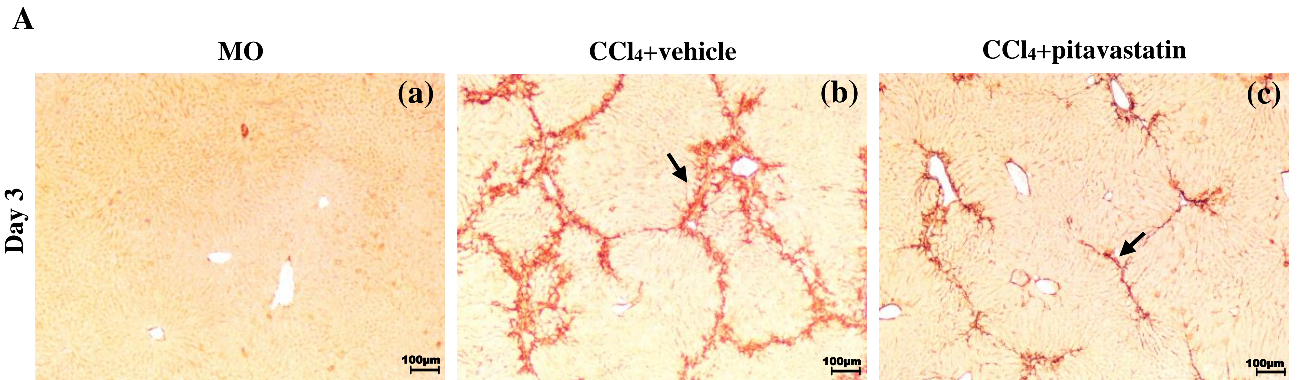


Figure 19. **The effect of pitavastatin treatment on collagen deposition in the liver.** (A) Representative images of Sirius Red staining for collagen fibers in the liver, indicated with an arrow, at 24 hours and 3 days after the last CCl_4 injection. Scale bar = 100µm. (B-C) Percentage fraction of collagen-positive signals quantified using ImageJ software. Data represented as (B) histograms and (C) side scatter plot. Data are expressed as mean ± SEM (n=9 for CCl_4+vehicle, 7 for CCl_4+pitavastatin mice for the 24 hours group, and n= 8 for CCl_4+vehicle, 10 for CCl_4+pitavastatin for the 3 days, and n=3 mice for MO group). ** $P < 0.005$; ***$P < 0.001$ (Mann-Whitney U test).

4. Pitavastatin Alters the Synthesis of α-SMA

We proceeded by immunohistochemistry of anti-α-SMA antibody on de-paraffinized tissue section obtained from C57BL/6 mice sacrificed 3 days after the last injection of CCl_4, as well as from MO mice. Noteworthy is that the necrosis obtained 24 hours after the CCl_4 injection makes the assessment of α-SMA problematic at day 1, with strong background due to the necrosis of the hepatocytes around the vessels. Thus, we performed our analysis for day 3 group only. Figure 20A shows an increase in the expression of α-SMA, as indicated by the black arrow, in the CCl_4-injected mice compared to MO mice. Quantification of the protein by ImageJ software indicated a significant increase in the synthesis of the α-SMA protein in CCl_4-injected mice (Area Fraction 12.1± 1.05%) compared to the control mice (Area Fraction 1.0 ± 0.05) at the indicated timepoint (Figure 20B). However, in the pitavastatin-treated mice, α-SMA protein levels significantly dropped by 35% compared to the CCl_4-injected mice (Figure 20B and C).



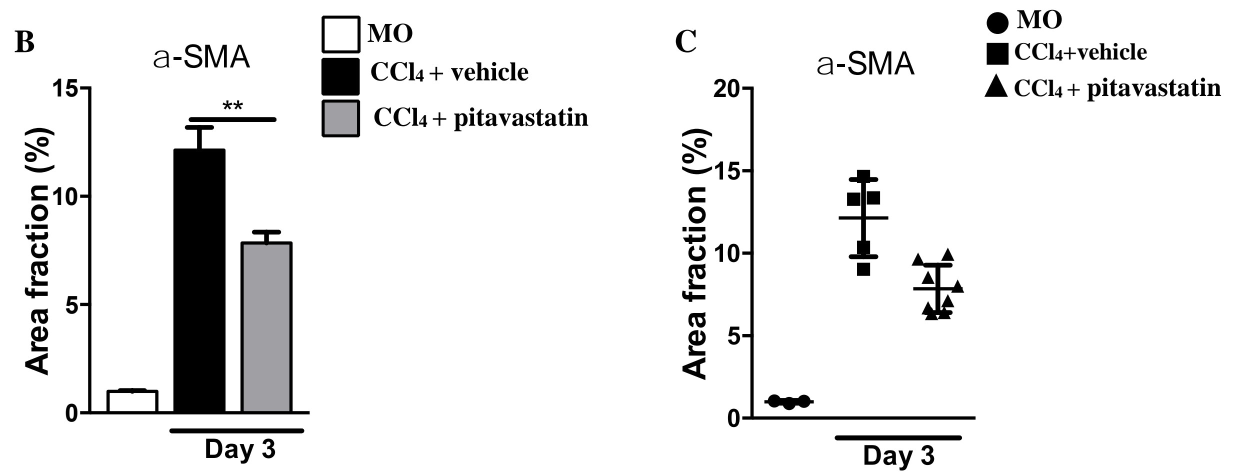


Figure 20. **Pitavastatin decreases the synthesis of α-SMA in the injured liver.** Immunohistochemistry was performed on different mouse liver tissues using α-SMA antibody. (a) Control liver section taken from control mice injected with mineral oil only. (b) Different portions of liver tissue taken from mice given injections of CCl_4 + vehicle. (c) Liver section taken from mice given injections of CCl_4+ pitavastatin. Scale bar = 100µm. (B-C) α-SMA synthesis was quantified using Image J software. Data represented as (B) Histogram and (C) Side scatter plot. Data are expressed as mean ± SEM (n= 5 for CCl_4+vehicle, 8 for CCl_4+pitavastatin for the 3 days group, and n=3 for MO). ** $P < 0.005$ (Mann-Whitney U test).

5. Effect of Pitavastatin on Fibrotic Gene Expression

In this context, we evaluated the effect of pitavastatin on the expression of several fibrotic genes. Figure 21 shows a tendency effect of 10mg/kg pitavastatin on the gene expression of α- SMA, a statistically significant effect on TGF-β, and no effect on PDGFRβ and CTGF (Figure 21A-C).

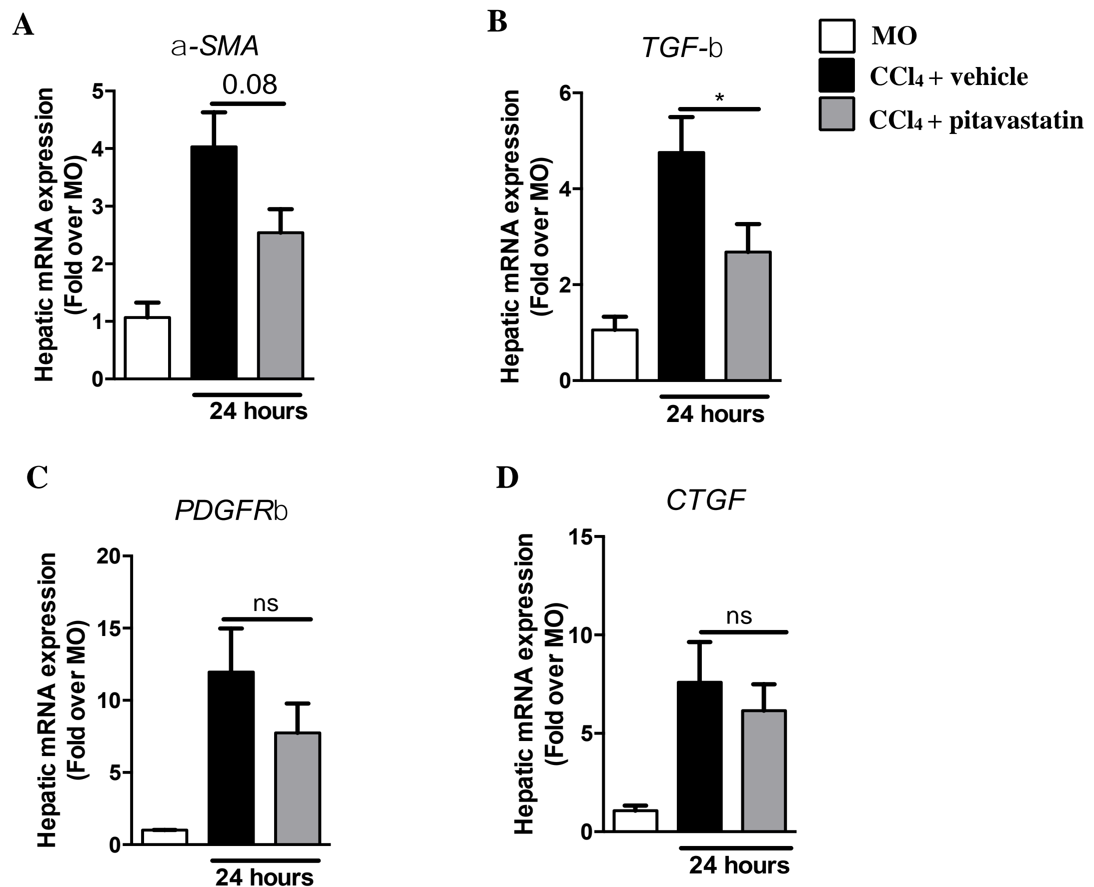


Figure 21. **Pitavastatin's effect on the expression of fibrotic genes**. RT-PCR was performed on RNA samples from control mice and mice given injections of CCl_4+vehicle or CCl_4+pitavastatin. mRNA levels of α-SMA, TGF-β, PDGFRβ and CTGF genes were analyzed at the 24 hours timepoint. 18S mRNA expression was used for normalization. Data are expressed as mean ± SEM (n=8 for CCl_4+vehicle, 10 for CCl_4+pitavastatin, and 3 for MO-injected mice). *$P < 0.05$; ns not significant (Mann-Whitney U test).

6. Effect of Pitavastatin on MMPs Gene Expression

It is well established that ECM degradative enzymes, such as MMPs, are promptly expressed by activated HSCs in response to chronic liver injury (Han, 2006). In this context, we aimed to assess the effect of pitavastatin on the mRNA levels of the profibrogenic MMP-2 and MMP-9. For the 24 hours group, there was a significant increase in MMP-2 and MMP-9 among the CCl_4-injected mice when compared to the MO group. Pitavastatin showed no effect on the expression of MMP-2 (Figure 22A), and a tendency to decrease the mRNA level of MMP-9 with a *P* value of 0.08 (Figure 22B).

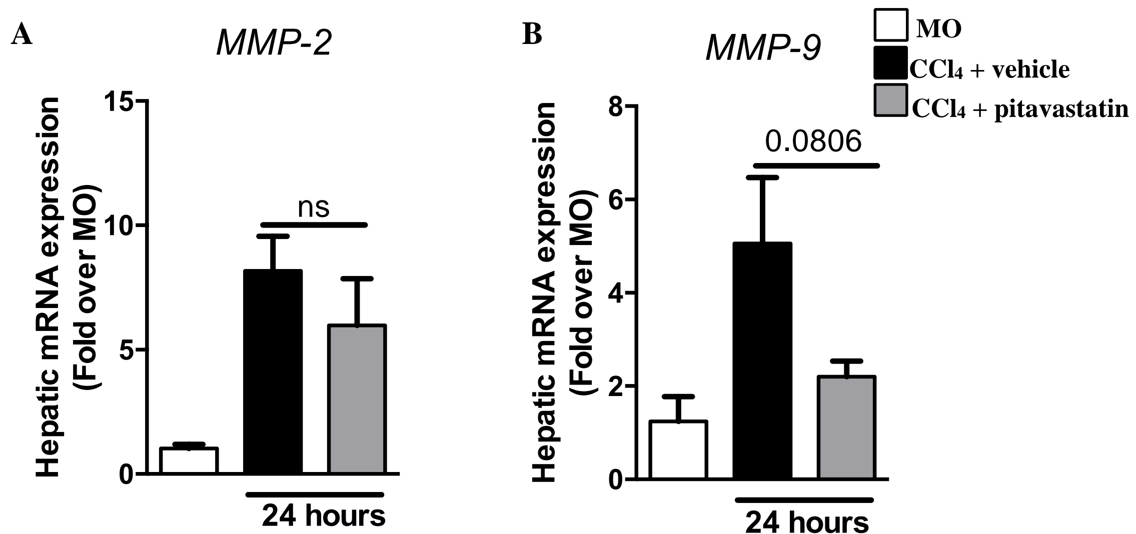


Figure 22. **The effect of pitavastatin treatment on expression MMPs genes**. RT-PCR was performed on control and treated mice with CCl_4+vehicle or CCl_4+pitavastatin. mRNA levels of MMP-2 and MMP-9 genes were analyzed at the 24 hours timepoint. 18S mRNA expression was used for normalization. Data are expressed as mean ± SEM (n=8 CCl_4+vehicle, 6 CCl_4+pitavastatin, and 3 for MO-injected mice). *$P < 0.05$; ns not significant (Mann-Whitney U test).

7. The Relation Between Pitavastatin and IL-6

In order to examine whether pitavastatin reduces the inflammatory response that occurs during chronic liver injury, we assessed the mRNA and protein levels of IL-6 cytokines. Gene expression analysis showed that IL-6 mRNA levels were reduced by 59% in the pitavastatin-injected mice compared to the CCl_4-injected mice (Figure 23A). After assessing the inhibitory effect of pitavastatin on the expression of IL-6 gene, the IL-6 protein was evaluated using ELISA. Mice sacrificed at both the 24 hours and 3 days timepoints were assessed for the modulation of IL-6 cytokine levels in the liver. For the 24 hours group, IL-6 synthesis is significantly increased in the CCl_4-injected mice compared to the MO group. However, there is a tendency of pitavastatin to inhibit the synthesis of IL-6 but the result is not significant, with a P value of 0.1066 (Figure 23B).

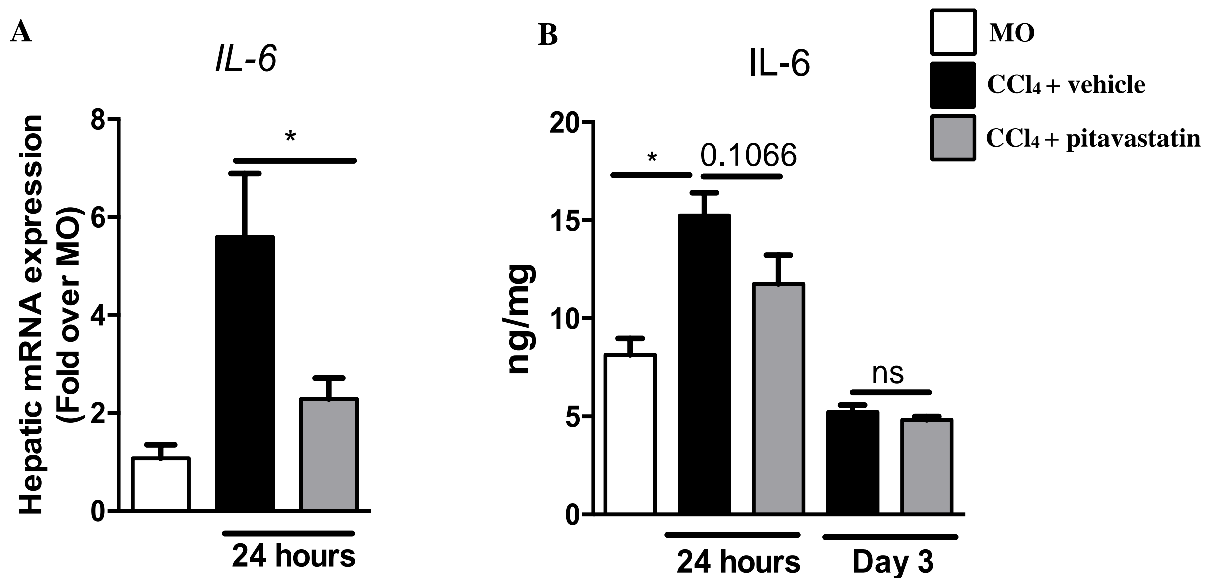


Figure 23. **Effect of pitavastatin on IL-6 levels in injured liver.** IL-6 gene expression and formation in the liver was measured by RT-PCR and ELISA, respectively. Measurement was done for the groups in the 24 hours and 3 days timepoints. Data are expressed as mean ± SEM (n=9 for CCl_4+vehicle, 7 for CCl_4+pitavastatin for the 24 hours group, and n= 8 for CCl_4+vehicle, 10 for CCl_4+pitavastatin for the 3 days group and n=3 mice in the MO group). * $P < 0.05$; ns not significant (Mann-Whitney U test).

C. Effect of Pitavastatin on Regression of Liver Fibrosis

1. Experimental Design

We next proceeded by assessing whether pitavastatin accelerates fibrosis regression in a CCl_4-induced liver fibrosis model. C57BL/6 mice were given injections of 0.6 ml/kg CCl_4 for 6 weeks, twice per week. 2 hours prior to the last injection of CCl_4, a group of mice were given i.p. injections of 10 mg/kg pitavastatin and the remaining mice were given an equivalent amount of DMSO, as a vehicle (Figure 24).

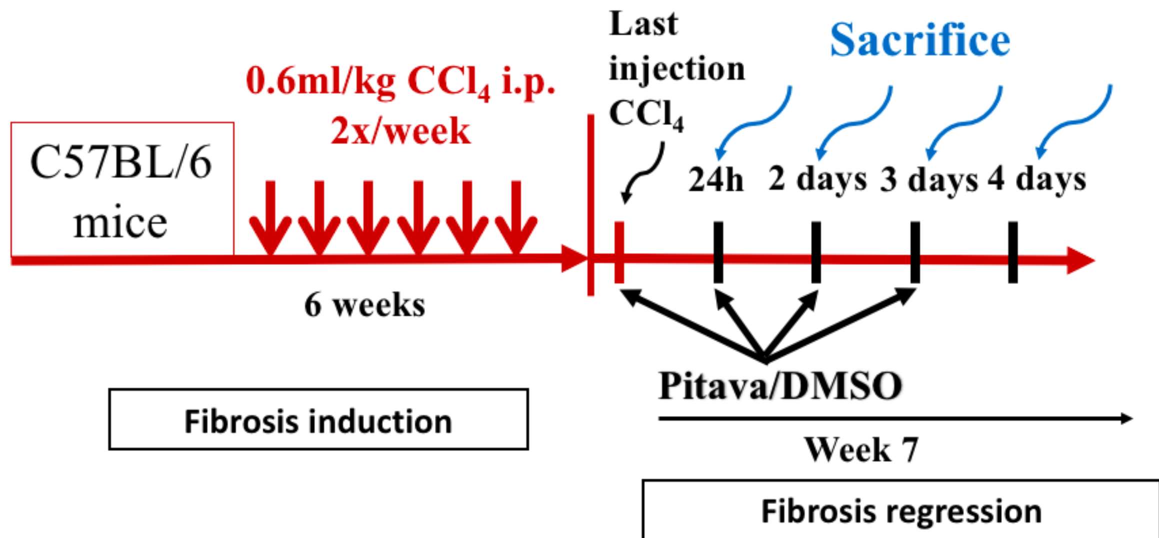
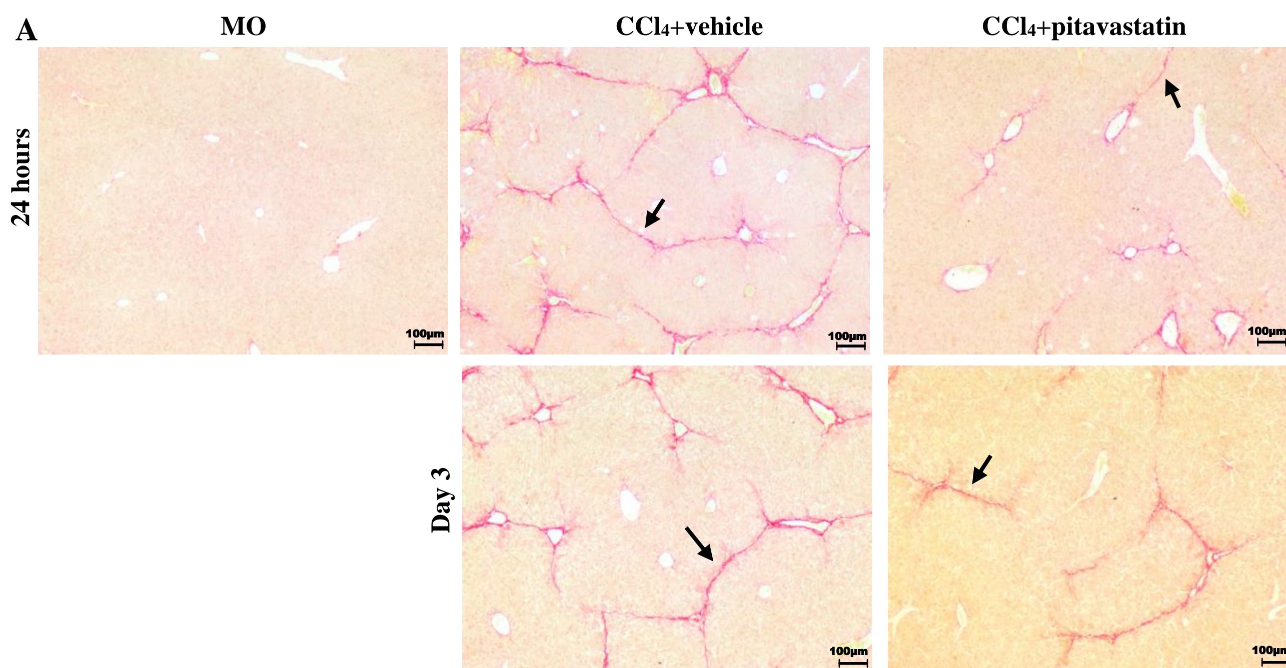


Figure 24. **Effect of pitavastatin on regression of liver injury.** C57BL/6 mice were given i.p. injections of CCl_4, and 5 mice were given MO injections, twice-weekly for 6 weeks. 10mg/kg pitavastatin or an equivalent amount of DMSO were administered daily after the last injection of CCl_4. Mice were grouped to be sacrificed on 1, 2, 3 and 4 days after the last injection of CCl_4.

The mice were sacrificed on 1,2,3, and 4 days after the last injection of CCl_4. 24 hours after the last injection of CCl_4, 18 mice (n=9 for CCl_4+vehicle and n= 9 for CCl_4+pitavastatin) as well as MO (n=5) were sacrificed. The other mice were injected daily with either pitavastatin or vehicle and sacrificed at days 2 (n= 8 for CCl_4+vehicle and 8 for CCl_4+pitavastatin), 3 (n= 7 for CCl_4+vehicle and 8 for pitavastatin), and 4 days after the final injection of CCl_4 (n= 8 for CCl_4+vehicle and n=9 for CCl_4+pitavastatin).

2. *Pitavastatin Reduces Deposition of Collagen in the Injured Liver*

To assess the effect of pitavastatin on the deposition and synthesis of collagen fibers during regression of liver fibrosis, liver sections were stained using Sirius Red. Figure 25A corresponds to representative images of liver sections from MO, CCl_4 + vehicle, and CCl_4 + pitavastatin-treated mice. The images show an increase in the Sirius Red staining in CCl_4-treated mice compared to MO mice at sacrificial days 1 and 3. Fibrosis was analyzed at day 1 and 3 after the last injection of CCl_4 in the absence (vehicle) or presence of pitavastatin. Figures 25B and 25C correspond to the percentage of area fraction of collagen, which show a statistically significant decrease in pitavastatin compared to CCl_4, from 4.2 ± 0.2 to 2.9 ± 0.1 and from 3.5 ± 0.2 to 2.8 ± 0.1 (Mean $\pm$ SEM) at days 1 and 3, respectively.



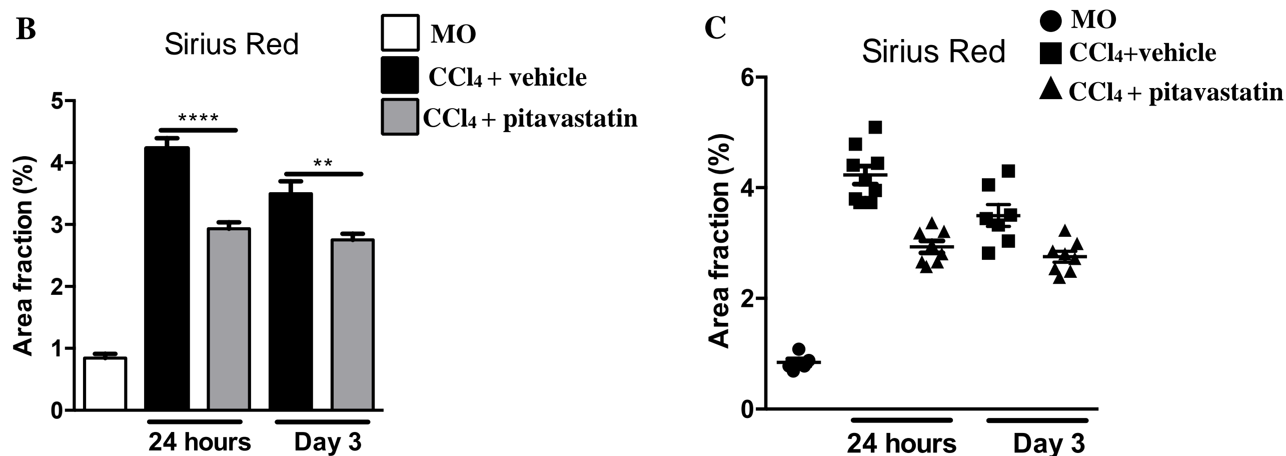
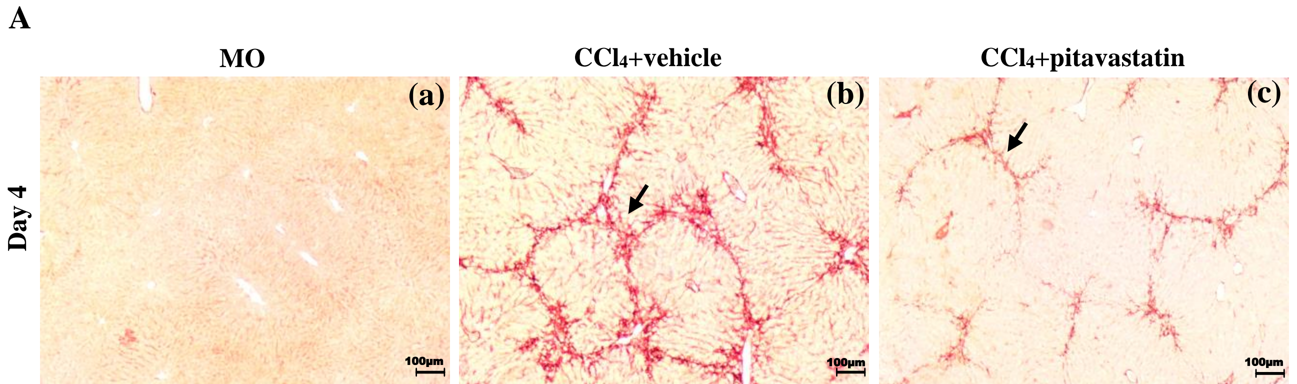


Figure 25. **The effect of pitavastatin treatment on collagen deposition in the liver.** (A) Representative images of Sirius Red staining for collagen fibers in the liver indicated with an arrow, at 24 hours and 3 days after the last CCl_4 injection. Scale bar= 100µm. (B-C) Area of collagen, quantified using ImageJ software. Data represented as (B) Histograms and (C) Side scatter plot. Data are expressed as mean ± SEM, (n=9 for CCl_4+vehicle, and 9 for CCl_4+pitavastatin for the 24 hours group), (n= 7 for CCl_4+vehicle, 8 for CCl_4+pitavastatin for the 3 days group), and (n=5 mice for MO). ** $P < 0.005$; ****$P < 0.0001$ (Mann-Whitney U test).

3. Pitavastatin Decreases the Synthesis of α-SMA During Regression of Liver Fibrosis

After identifying the effect of pitavastatin on collagen deposition, we determined whether this effect is also associated with myofibroblast activation. Immunohistochemistry of α-SMA was performed on liver sections from CCl_4+vehicle, CCl_4+pitavastatin, and MO mice. Figure 26A shows that at day 4, myofibroblast activation, assessed by α-SMA, was increased in the CCl_4 compared to MO-injected mice as indicated by the black arrow. Furthermore, quantification of this protein by ImageJ software indicated a significant increase in the synthesis of α-SMA in CCl_4+vehicle group compared to the control mice at the indicated timepoint. However, in the pitavastatin-treated mice, α-SMA protein is significantly decreased compared to the CCl_4-injected mice, with the data showing a decrease from 7.5 ± 0.4 to 5.0 ± 0.3 (Mean ± SEM) at day 4 (Figure 26B and C).



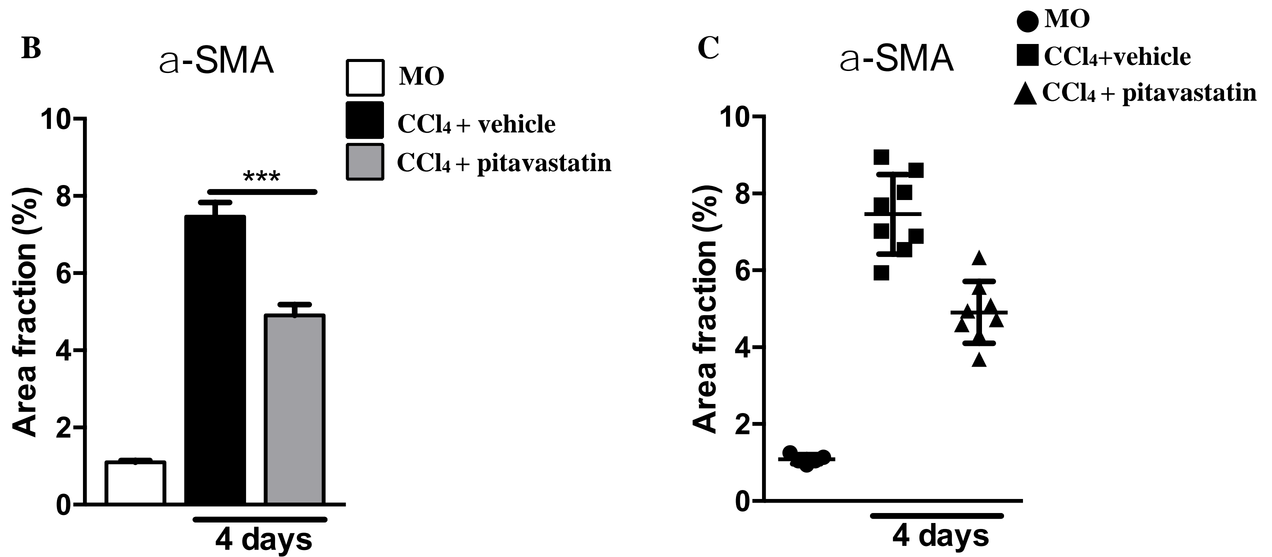


Figure 26. **α-SMA decreases during regression after the administration of 10 mg/kg pitavastatin for 4 days.** (A) Representative images are shown for MO, CCl_4+vehicle, and CCl_4+pitavastatin-injected mice. Scale bar=100 µm. (B-C) Quantification of histological changes of α-SMA percent area using Image J software. Data represented as (B) Histograms and (C) Side scatter plot. Data are expressed as mean ± SEM, (n=8 for CCl_4+vehicle and CCl_4+pitavastatin and 5 for MO). $***P < 0.001$ (Mann-Whitney U test).

3. Effect of Pitavastatin on Fibrotic Genes During Regression of Fibrosis

In order to understand the effect of pitavastatin on gene expression of some fibrotic genes during cessation of liver injury, RT-PCR was performed for control, CCl_4+vehicle, and CCl_4+pitavastatin mice. The results obtained show that pitavastatin triggers a significant decrease in the mRNA levels of the α-SMA, PDGFRβ genes (Figure 27A and C) compared to CCl_4 at the 24 hours timepoint, with the data showing a 2-3-fold decrease for both genes. No effect was obtained on the expression of TGF-β and CTGF genes (Figure 27B and D).

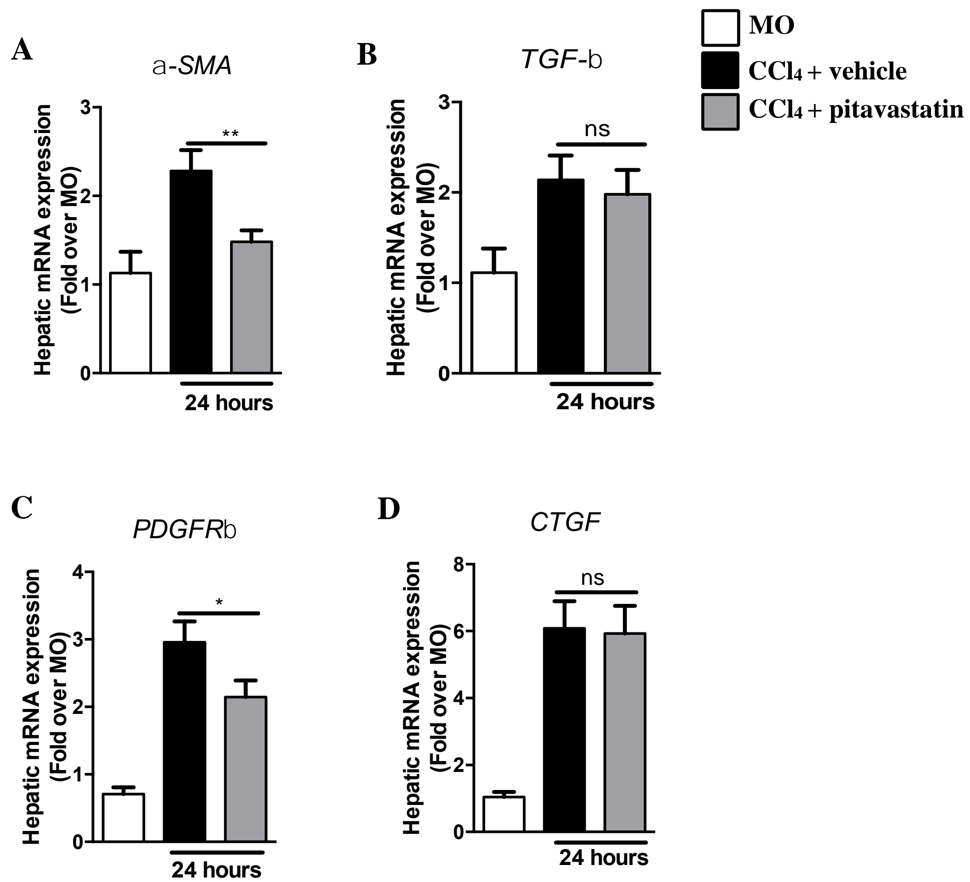


Figure 27. **Changes in fibrotic gene expression after administration of pitavastatin.** mRNA levels of α-SMA, PDGFRβ, TGF-β, and CTGF genes were analyzed at the 24 hours timepoint. 18S mRNA expression was used for normalization. Data are expressed as mean ± SEM (n=9 for CCl_4+vehicle and CCl_4+pitavastatin groups and n=5 for MO-injected mice). **P* < 0.05; ***P* < 0.005; ns not significant (Mann-Whitney U test).

4. Effect of Pitavastatin on MMPs and TIMP-1 Genes After Cessation of Injury

In addition to the fibrotic genes, we also examined the pitavastatin effect on hepatic mRNA expression of MMP-2, MMP-9, and TIMP-1 genes for the mice sacrificed 24 hours after the last injection of CCl_4. Gene expression analysis showed significant reduction in the expression levels of the MMP-2 gene, from 2.7 ± 0.4 fold in the CCl_4-injected mice to 1.6 ± 0.2 fold (Mean ± SEM) in the pitavastatin-injected mice (Figure 28A). However, when analyzing MMP-9 and TIMP-1 mRNA levels, pitavastatin showed no effect on their expression (Figure 28B and C)

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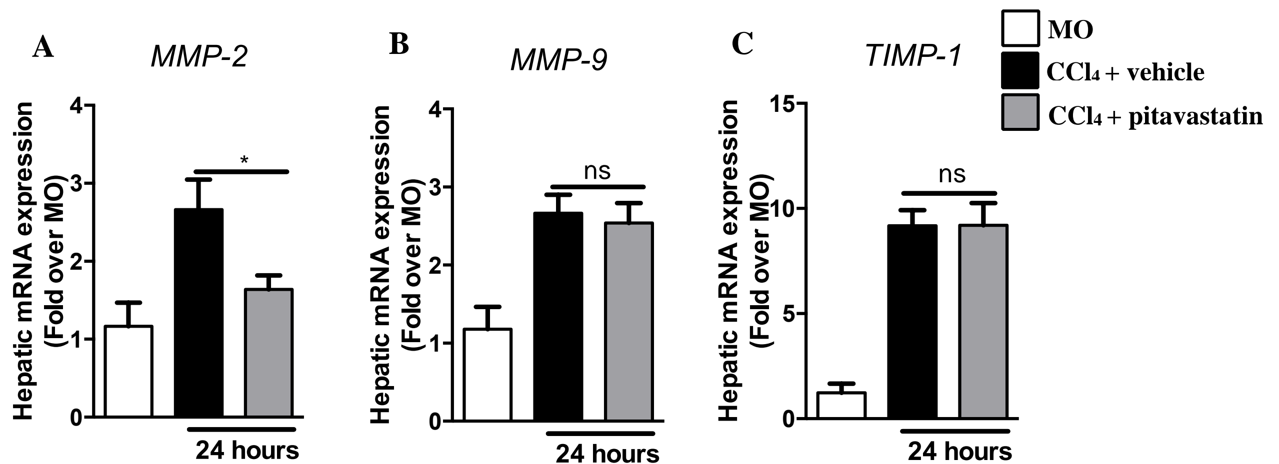


Figure 28 . **The effect of pitavastatin treatment on the expression of MMPs and TIMP-1 genes**. RT-PCR was performed on RNA samples from control mice and mice treated with CCl_4+vehicle or CCl_4+pitavastatin. mRNA levels of MMP-2, MMP-9 and TIMP-1 genes were analyzed at the 24 hours timepoint. 18S mRNA expression was used for normalization. Data are expressed as mean ± SEM (n=9 for CCl_4+vehicle and CCl_4+pitavastatin and n=5 for MO). *$P < 0.05$; ns not significant (Mann-Whitney U test).

5. *Pitavastatin's Effect on IL-6 Gene Expression and Protein Formation*

Having identified evidence of a role of pitavastatin in reducing fibrosis, we aimed to determine whether pitavastatin activity involves the inflammatory pathway during regression of fibrosis. The expression of IL-6 gene and its protein formation in the liver were assessed using RT-PCR and ELISA, respectively. For the 24 hours group, IL-6 mRNA expression levels and protein synthesis significantly increase in the CCl_4-injected mice compared to the MO group. However, there is no effect of pitavastatin on the inhibition of IL-6 expression at both the gene and protein levels compared to the CCl_4 mice.

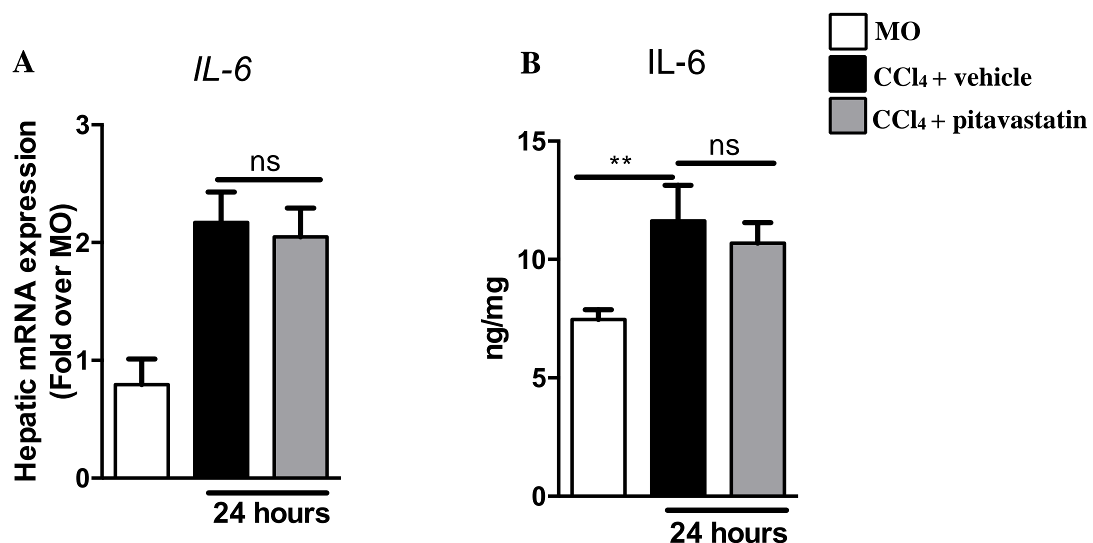


Figure 29. **Effect of pitavastatin on IL-6 cytokine levels during regression of liver injury**. IL-6 gene expression and formation in the liver was measured by RT-PCR and ELISA, respectively. Measurement was done for the groups in the 24 hours timepoint. Data are expressed as mean ± SEM (n=9 for each of CCl_4+vehicle and CCl_4+pitavastatin groups and n=5 for MO-injected mice). ** $P < 0.005$; ns not significant (Mann-Whitney U test).

6. *Dynamic Changes of Intrahepatic Macrophage Subsets During Regression from Liver Injury*

Macrophage infiltration into the liver upon chronic injury has been linked to the progression of liver inflammation and fibrosis (Duffield et al., 2005). Restorative macrophages (Ly6C^{low}) have been identified to functionally contribute to the regression of liver fibrosis (Ramachandran et al., 2012). To characterize the intrahepatic macrophages in our model, liver leukocytes were isolated and subjected to multicolor flow cytometry analysis. Gating strategy is represented in Figure 30. Intrahepatic macrophages were defined as living, CD45^{+} cells that stain positive for myeloid marker (CD11b), intermediate or high for the macrophage marker (F4/80), and negative for the T cell (TCRβ), B cell (CD19) and neutrophil (Ly6G) markers and were further divided into Ly6C^{high} and Ly6C^{low} expressing subpopulations.

During liver fibrosis regression, Ly6C^{high} macrophages predominate in the CCl_4-treated mice at days 2 and 3 after the last injection of CCl_4 (Figure 31A and C). The relative composition of intrahepatic macrophages changed significantly during regression upon the administration of 10mg/kg pitavastatin, with a progressive increase of the Ly6C^{low} macrophage subset mirrored by a decrease in the Ly6C^{high} population (Figure 31 B and D).

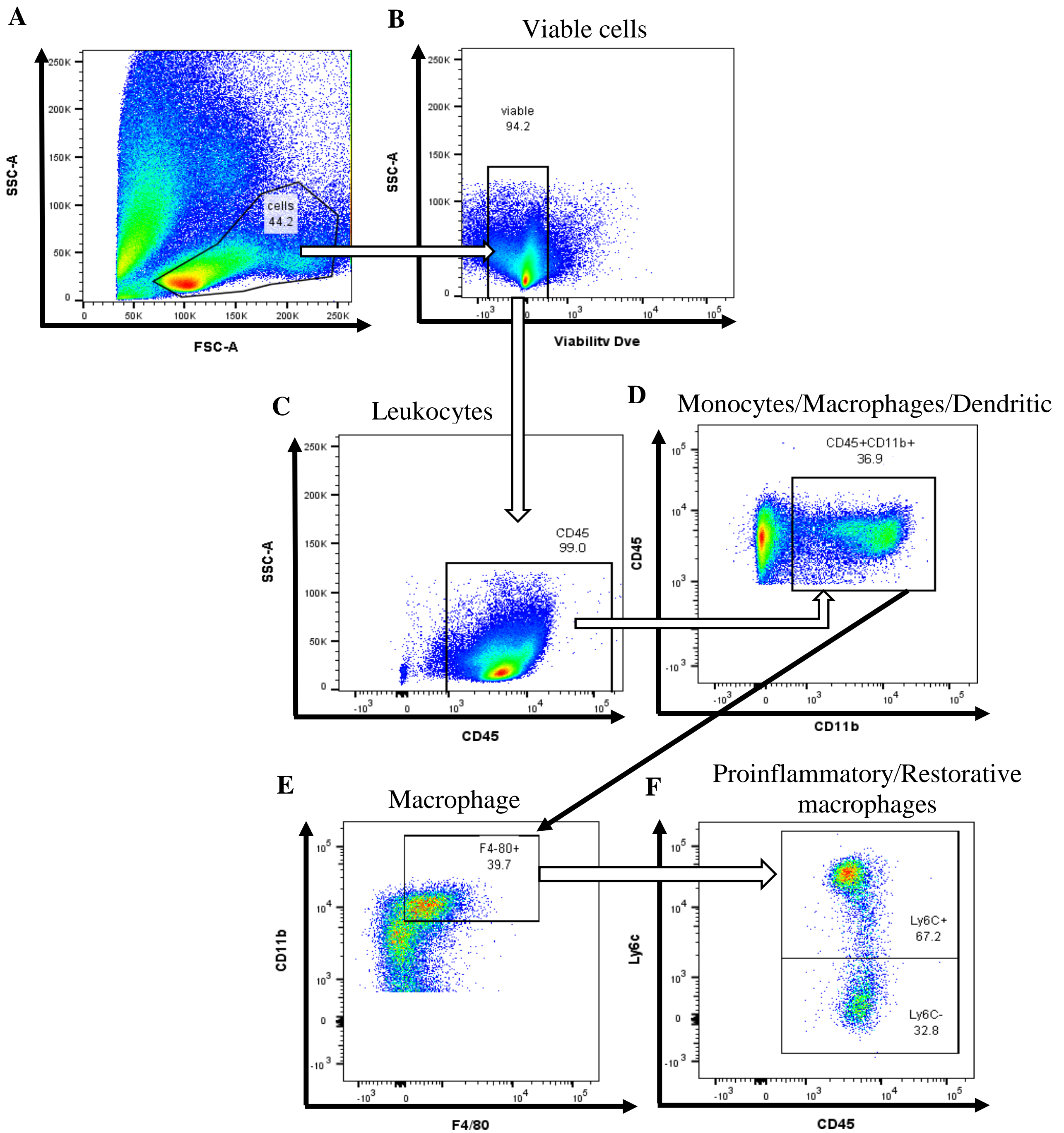
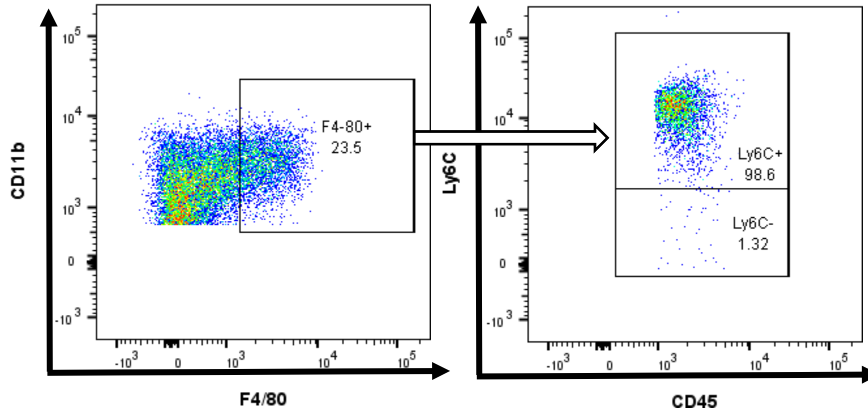


Figure 30. **Gating strategy to identify hepatic macrophages**. (A) Leukocytes were gated on a forward (FSC)/side scatter (SSC) plot. (B) Viable cells were selected by Live/Dead viability dye exclusion. (C) Live cells were further gated on CD45-positive cells. (D) Cells positive for TCR-β, CD19, and Ly6G were excluded from subsequent macrophage gating and $CD11b^+$ cells were selected. (E) Macrophages were selected as viable cells $CD45^+$ TCR-β^- $CD19^-$ $Ly6G^-$ and $CD11b^+$ $F4/80^+$. Representative flow cytometry plots are shown.

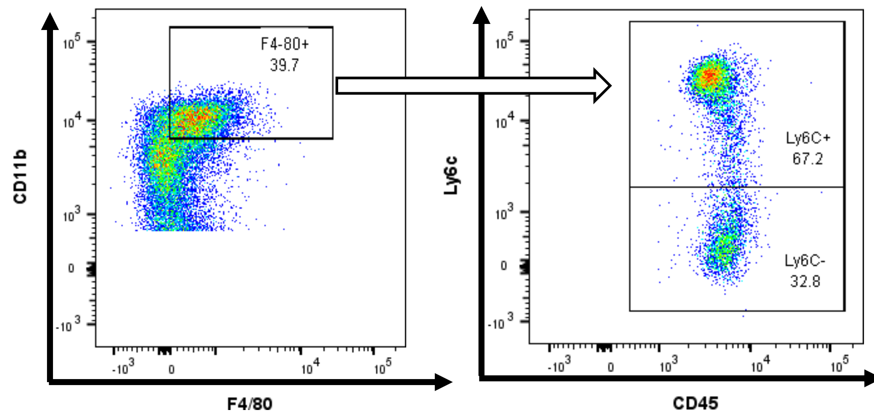
A

Day 2: CCl_4+vehicle-treated mice

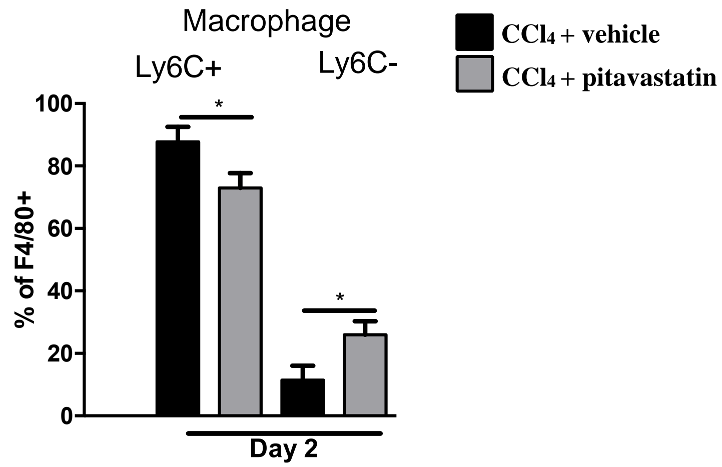


B

Day 2: CCl4+pitavastatin-treated mice

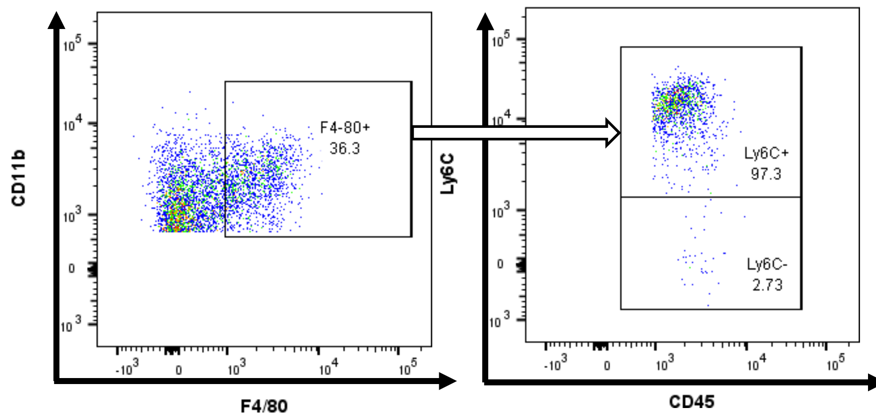


C



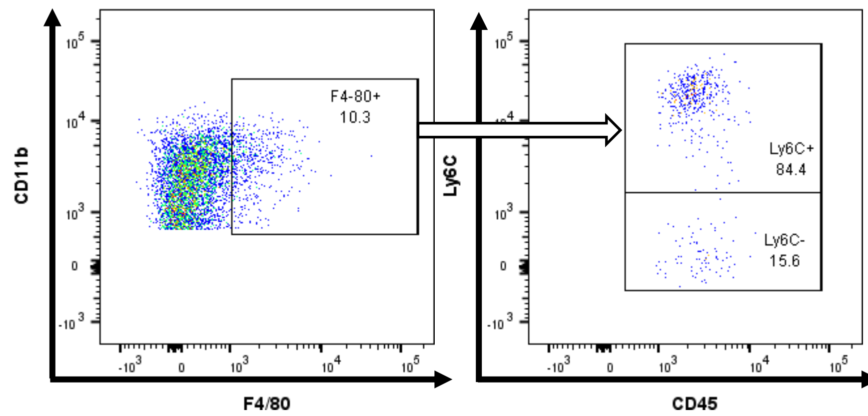
D

Day 3: CCl4+vehicle-treated mice



E

Day 3: CCl4+Pitavastatin-treated mice



F

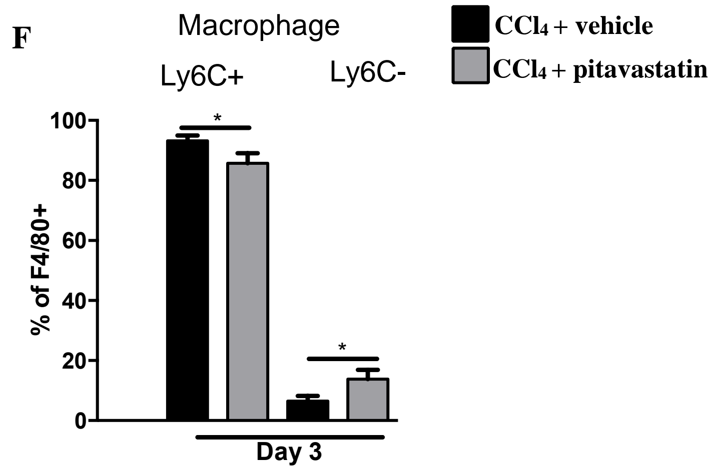


Figure 31. **Flow cytometry analysis of intrahepatic leukocytes.** Intrahepatic leukocytes were isolated from the liver of (A-D) CCl_4+vehicle or (B-E) CCl_4+ pitavastatin-treated mice and stained using a combination of selective antibodies. Macrophages were characterized as $Live^+/CD45^+/Ly6G^-/TCR\text{-}\beta^-/CD19^-/CD11b^+/F4/80^+$ and quantified by FACS. Macrophage subpopulations were further subdivided into $Ly6C^{high}$ and $Ly6C^{low}$ subsets. (C-F) Percentage of the stated macrophage subsets at the indicated timepoints (expressed as a percent of total macrophage cells). Data are expressed as mean ± SEM (n=8 for CCl_4+vehicle, 8 for CCl_4+pitavastatin-injected mice for day 2), and (n= 7 for CCl_4+vehicle,8 for CCl_4+pitavastatin-injected mice for day 3). * $P < 0.05$ (Mann-Whitney U test).

CHAPTER VI

DISCUSSION AND PERSPECTIVES

In our study, we first confirmed that CCl_4 increased fibrosis in the liver by increasing collagen deposition and α-SMA production. We also demonstrated that CCl_4 up-regulated the expression of genes that are highly associated with the modulation of ECM, including CTGF, α-SMA, TGF-β, and TIMP-1. CCl_4 also up-regulated the expression of inflammatory genes CCL2, CCL3 and CCL4. Additionally, the deleterious effect elicited by CCl_4 on the liver was evaluated by two liver enzymes, ALT and AST, wherein high levels of these enzymes were indicative of an injured liver.

1. Pitavastatin with Antifibrotic Effects.

In principle, antifibrotic drugs can mediate their effects on three different levels, a) inhibition of hepatic stellate cell activation, (b) reduction of fibrogenic cell accumulation by growth inhibiting or proapoptotic compounds, and/or (c) suppression of extracellular matrix synthesis or enhancement of its degradation (Lotersztajn et al., 2005). In this context, this study attempts to elucidate the molecular mechanisms underlying the beneficial effects of pitavastatin on hepatic inflammation and fibrogenesis in a murine model. In previous studies, our group and others have shown that statins such as lovastatin, fluvastatin, and simvastatin inhibit rat and human HSC proliferation *in vitro* (Mouawad et al., 2016; Rombouts et al., 2003). Our group had also reported that the inhibition of hepatic myofibroblast proliferation by statins depends on cyclooxygenase-2 (COX-2) (Mouawad et

al., 2016). The present study revealed that pitavastatin reduces collagen deposition, α-SMA synthesis by activated HSCs, and the expression of TGF-β, and showed a trend to decrease α-SMA gene expression in the liver. These observations aligned with prior studies that showed that atorvastatin, another member of the statin family, attenuated hepatic fibrosis in rats after bile duct ligation (Trebicka et al., 2010; Klein et al., 2012). TGF-β has a pivotal role in the initiation, promotion, progression, and transdifferentiation of HSCs into hepatic myofibroblasts. Concomitant with increased activity of TGF-β during fibrogenesis, HSCs increase production and deposition of collagen leading to progressive scarring and loss of organ function (Sanderson et al., 1995). Thus, the reduction in the activity of myofibroblasts by pitavastatin was associated with the reduction of TGF-β synthesis, and perhaps signaling, which prevents ongoing liver fibrosis in the toxic-liver injury model. Interestingly, it was shown that the growth inhibitory effects of several factors, including TNF-α and endothelin-1, on human hepatic myofibroblasts involve the induction of COX-2 and a subsequent generation of PGE_2 (Gallois et al., 1998). Together, these data point to COX-2 as a source of antifibrogenic prostaglandins in the liver, the beneficial effects of which include the inhibition of hepatic myofibroblast proliferation and collagen synthesis, as shown in culture studies (Beno, Espinal, Edelstein, & Davis, 1993). In this context, we are planning to assess the levels of COX-2 and PGE_2 in the livers from our existing experiments in order to unravel the molecular mechanism behind the inhibitory effect shown in this study. Furthermore, studies using cleaved caspase-3 analysis by immunohistochemistry should be conducted to investigate whether pitavastatin decreases the apoptosis of hepatocytes.

2. What is the role of the inflammatory pathway?

Chronic inflammation within the liver is tightly linked to fibrosis in virtually all types of liver disease and in experimental models of NASH and liver fibrosis (Seki & Schwabe, 2015). Based on the complexity of hepatic fibrosis, the possibilities for therapeutic targeting are quite heterogeneous. Our group had previously studied the effect of statins on human monocytic cells, where they demonstrated that simvastatin decreases the expression of LPS-induced COX-2 and they verified that the inhibition is in a Rac and NF-κB dependent manner (Habib et al., 2007). Additionally, our preliminary data points to pitavastatin as having the most promising effect in inhibiting proinflammatory cytokines in the macrophages stimulated by LPS, when compared to other statins (Figure 10). In the present study, we demonstrated the ability of pitavastatin to decrease the production of IL-6, supporting that pitavastatin reduces fibrosis by inhibiting inflammation and targeting innate immune cells, mainly macrophages. Overall, the perpetuation of inflammation in the liver appears to counteract the degradation of ECM, thus leading to fibrosis progression. Additional evaluation of the level of the intrahepatic macrophages by immunohistochemistry (F4/80$^+$ cells detection) will be conducted. To further support the importance of inflammation in the fibrogenic process, our group has shown that statins induce heme-oxygenase-1 (HO-1), an anti-inflammatory enzyme, expression in a NO-dependent manner (Mouawad et al., 2013). Moreover, it has been demonstrated that HO-1 expression plays an antifibrogenic role in human hepatic myofibroblasts by inhibiting their proliferation and their synthesis of collagen (Li et al., 2003; Li et al., 2004). IL-10 has been described as an anti-inflammatory cytokine, which is upstream of HO-1. A small pilot trial of interleukin-10 in 24 patients with chronic hepatitis C showed improvement of

inflammation and was associated with a decrease in fibrosis (Nelson, Lauwers, Lau, & Davis, 2000). Therefore, our study will further assess the effect of statin on the production of IL-10 and HO-1 expression in the liver.

Additionally, *in vivo* studies showed that simvastatin decreased inflammation and fibrosis by inhibiting RhoA and Ras downstream, whereas RAC1 inhibition showed no effect (Schierwagen et al., 2016). In this context, a further study using the ROCK inhibitor (Fasudil, HA-1077) will be conducted in order to assess its effect on liver inflammation and fibrosis.

3. *Macrophages during the Regression of Liver fibrosis.*

The view on macrophages in the liver has drastically changed in the past years, as it became apparent that hepatic macrophages consist of a heterogeneous population that is implicated in liver health and disease (Ju & Tacke, 2016). Based on experimental and clinical data linking the recruitment of monocyte-derived inflammatory macrophages in the liver to the progression of NASH and fibrosis (Zimmermann & Tacke, 2011), we next hypothesized that pharmacological inhibition of HMG-CoA reductase in macrophages could represent a successful therapeutic approach that enhances fibrosis regression during chronic liver injury *in vivo.* In our study, after the discontinuation of injury, the inhibition of the proinflammatory macrophages by pitavastatin was associated with reduced fibrosis and acceleration of regression, where we showed that pitavastatin reduces Ly6C^{high} macrophage, and shifts the macrophage population toward the Ly6C^{low} phenotype. It has been previously described that monocyte-derived cells can activate HSCs in the context of

chronic liver injury in mice and humans by secreting fibrotic factors such as fibrosis-related genes TGF-β or PDGF (Karlmark et al., 2009; Zimmermann et al., 2010). In this context, we demonstrated that, during regression, pitavastatin downregulates the gene expression of α-SMA and PDGFRβ, reduces the synthesis of α-SMA protein by myofibroblasts, and the Sirius Red staining, in parallel with the reduction of the proinflammatory macrophages $Ly6C^{high}$. It was also well characterized that restorative macrophages are associated with the up-regulation of MMP-9 and MMP-12, which are implicated in scar-resolution (Ramachandran et al., 2012). In contrast, MMP-2 is believed to be implicated in the degradation of normal ECM and progression of fibrosis (Han, 2006). In our study, MMP-9 gene expression remained highly expressed, whereas MMP-2 was significantly down-regulated upon pitavastatin administration. Thus, the switch to a restorative macrophage phenotype confers a number of resolution features, highlighting the importance of macrophages in liver fibrosis regression.

In line with our finding, it has also been shown that the administration of distinct macrophage subsets, especially subpopulations with $Ly6C^{low}$ expression, can improve regression of liver fibrosis (Thomas et al., 2011). On the other hand, adoptive transfer of immature $Ly6C^{high}$ bone marrow-derived monocytes aggravated experimental liver fibrosis (Karlmark et al., 2009). Moreover, our results showed that pitavastatin accelerated the regression of liver fibrosis after cessation of CCl_4, where we have shown that pitavastatin can lead to the reduction of collagen and α-SMA protein deposition, and down-regulation of PDGFRβ, α-SMA, and MMP-2 gene expression after one injection of pitavastatin**.** However, it remains unclear whether a lower dosage of statin might confer a lower

inhibitory effect at day 1 compared to day 4, and therefore further experiments could clarify the effect of pitavastatin on the acceleration of liver regression by assessing the effect within the first 24 hours post CCl_4 injection. Additional experiments are required to better distinguish between the antifibrotic and the acceleratory effect of pitavastatin, which could be achieved by lower doses. A schematic overview of the effect of pitavastatin on different players is shown in Figure 32.

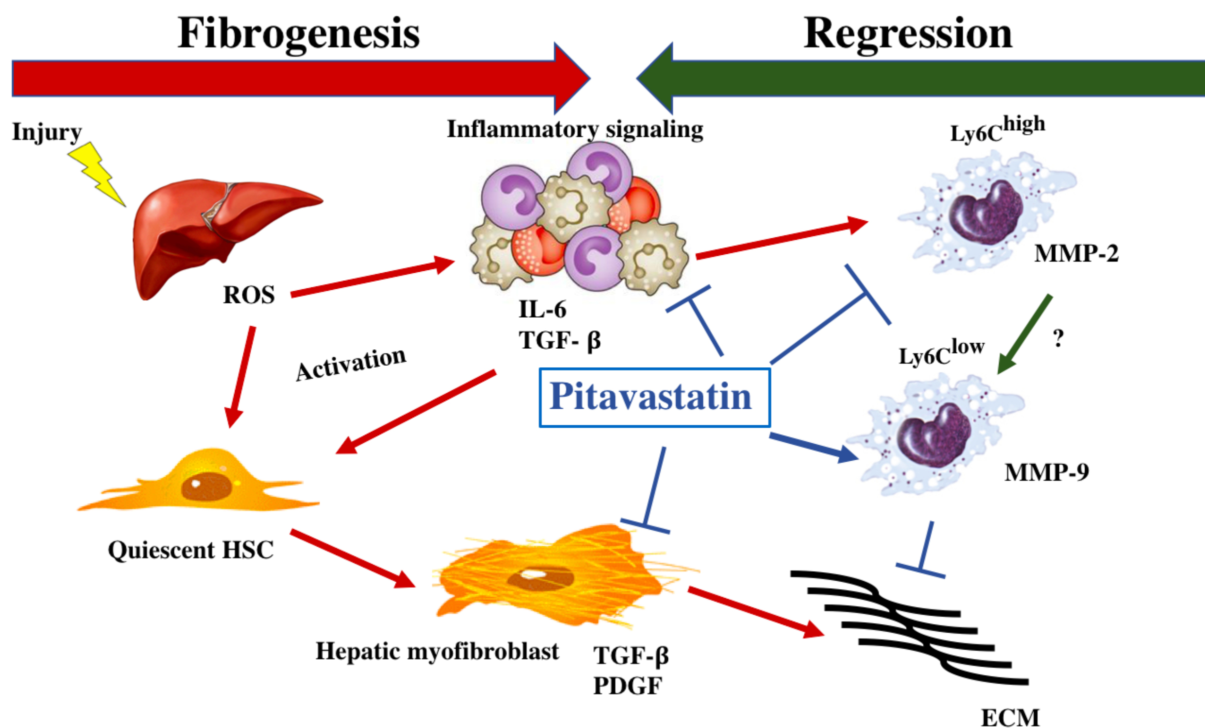


Figure 32. **Schematic overview of the effect of pitavastatin on inflammation and fibrogenesis**.

Further studies are required, including the depletion of macrophages using liposome chlodronate, in order to highlight the role of macrophages in this process. In addition, investigations on the role of other intrahepatic leukocytes, including T cells or NK cells, are recommended. Moreover, to exclude the possibility that model-specific effects had confounded the above-mentioned results, we are planning to repeat the experiment using another model of chronic liver injury, methionine-choline deficient (MCD) diet, that results in severe steatohepatitis.

Overall, inhibiting the inflammatory macrophages in the liver, as defined in our study, makes pitavastatin an interesting drug for the treatment of liver diseases (e.g. NASH).

In conclusion, our study demonstrates that treatment with pitavastatin attenuates the development of liver fibrosis, and accelerates its regression, through multiple mechanisms *in vivo*, including inhibition of TGF-β, and PDGFRβ. We further showed that pitavastatin not only targets hepatic myofibroblasts, but also macrophages by shifting the macrophage population towards Ly6C^{low} cells. In summary, statins are promising agents due to the multiple target effects for the treatment of chronic liver injury. Therefore, a combination of drugs with distinct antifibrogenic actions may result in therapeutic benefits at low doses and reduce the risk of unwanted side effects.

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