Cytokines and Cytokine-Receptors in the Pathogenesis of Alcoholic Hepatitis

Silvia Affò

,

UNIVERSITAT DE BARCELONA

IDIBAPS
Institut D'Investigacions Biomèdiques August Pi i Sunyer

CYTOKINES AND CYTOKINE-RECEPTORS

IN THE PATHOGENESIS OF ALCOHOLIC HEPATITIS

Presented by

SILVIA AFFÒ

For the degree of Doctor in Medicine

Work performed under the direction of **Dr. Pau Sancho Bru** and **Dr. Ramón Bataller Alberola**, in the laboratory of Hepatic Fibrosis, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Department of Hepatology, Hospital Clínic of Barcelona.

Silvia Affò
Ph.D Student Candidate

Dr. Pau Sancho Bru
Dr. Ramón Bataller Alberola
Directors

Ph.D. Program of Medicine
2014

Learn from yesterday, live for today, hope for tomorrow.
The important thing is not to stop questioning.

Albert Einstein

ABIC	Age, Serum Bilirubin, INR, and serum Creatinine score
ADH	Alcohol Dehydrogenase
AH	Alcoholic Hepatitis
AHHS	Alcoholic Hepatitis Histological Score
AKI	Acute Kidney Injury
ALD	Alcoholic Liver Disease
ALDH2	Aldehyde Dehydrogenase 2
AMPK	Adenosine Monophosphate-activated Protein Kinase
APAP	Acetaminophen
APCs	Antigen-presenting Cells
Bambi	Bone Morphogenic Protein and Activin Membrane-Bound Inhibitor
CCl_4	Carbon Tetrachloride
CYP2E1	Cytochrome P450 2E1
DC	Dendritic Cell
DCC Diet	3,5-dietoxycarbonyl-1-1, 4-dihydrocollidine diet
DF	Discriminant Function
ECM	Extracellular Matrix
ERK	Extracellular Signal-regulated Kinase
FasL	Fas Ligand
HCC	Hepatocellular carcinoma
HSCs	Hepatic Stellate Cells
ICAM-1	Intracellular Adhesion Molecule-1
IFN-γ	Interferon-γ
IKK	IkappaB Kinase
IL	Interleukin
INR	International Normalized Ratio
IRAK	Interleukin-1 Receptor-associated Kinase
IRF	Interferon Regulatory Transcription Factor
JAK	Janus Kinase
JNK	c-Jun N-terminal Kinases
KC	Kupffer Cells
LPC	Liver Progenitor Cells

LPS	Lipopolysaccharide
MAPK	Mitogen-activated Protein Kinase
MCP-1	Monocyte Chemotactic Protein-1
MELD	Model for End-Stage Liver Disease
MIP-3α	Macrophage Inflammatory Protein-3 α
MMPs	Metalloproteases
MPO	Myeloperoxidase
MyD88	Myeloid differentiation primary response gene (88)
NAFLD	Non-alcoholic Fatty Liver Disease
NASH	Non-alcoholic Steatohepatitis
NF-Kb	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
NK	Natural Killer Cells
NKT	Natural Killer T cells
NOS2	Nitric Oxide Synthase, inducible
PAF	Platelet-activating Factor
PAMPs	Pathogen-associated Molecular Patterns
PGE_2	Prostaglandin E2
PDFG	Platelet-derived Growth Factor
PI3K	Phosphoinositol 3-kinase
PMN	Polymorphonuclear cells
PPAR	Peroxisome proliferator-activated receptor
PPRs	Pattern Recognition Receptors
ROS	Reactive Oxygen Species
shRNA	Small-hairpin RNA
SIRS	Inflammatory Response Syndrome
STAT	Signal Transducer and Activator of Transcription
TGF-β	Transforming Growth Factor Beta
Th-1	T-helper Cells Type 1
Th-2	T-helper Cells Type 2
Th-17	Interleukin-17 Producing T Cells
TIMPs	Tissue Inhibitor of Metalloproteinases
TLRs	Toll-like Receptors
TNF	Tumor Necrosis Factor

TNFR Tumor Necrosis Factor Receptor

TRAIL TNF-related Apoptosis-inducing Ligand

TRAF TNF Receptor-associated Factors

TRIF TIR-domain-containing Adapter-inducing Interferon-β

Treg Regulatory T Cells

TWEAK TNF-like Weak Inducer of Apoptosis

VEGF Vascular Endothelial Growth Factor

FIGURES AND TABLE INDEX

INTRODUCTION

1. ALCOHOLIC LIVER DISEASE

Alcoholic liver disease (ALD) is a major cause of morbidity and mortality worldwide and presents a broad spectrum of diseases ranging from fatty liver and hepatic inflammation, to more severe forms of liver injury, including alcoholic hepatitis (AH), cirrhosis and hepatocellular carcinoma (HCC) (Altamirano et al. 2011; Gao et al. 2011). Excessive alcohol intake also favors the progression of other liver diseases, such as chronic viral hepatitis (hepatitis B and C) and other metabolic liver diseases, such as hemochromatosis, Wilson disease and fatty liver associated with the metabolic syndrome (Clouston et al. 2007; Singal et al. 2011). Moreover, alcohol abuse can lead to severe damage in the nervous system, heart, kidney and pancreas in addition to psychiatric manifestations.

According to the World Health Organization, alcohol consumption is responsible for about 3.2% of global mortality and for about 4% of life years lost (Organization Wh. The World Health Report 2002). Alcohol consumption is linked to more than 60 disease conditions, indicating that it affects practically every organ. Among totally alcohol attributable diseases, ALD is the main cause of death in the adult population. Among 29 million EU citizens, 6% present chronic liver diseases, the 5th most common cause of death. While there is a clear reduction in new cases of hepatitis B and C, the number of patients with alcoholic and non-alcoholic steatohepatitis is constantly increasing and the majority of patients with end-stage liver disease in the EU in the coming decades will be related to alcohol abuse. Liver diseases represent the main source of the burden attributable to alcohol with 28.3% of total mortality, followed by traffic accidents 26.2% and several types of cancer 21%.

Despite the profound economic and health impact of ALD and its severe effects on health (McCullough et al. 1998), few advances in the management of patients with this condition have been made (Tome et al. 2004). The lack of modern diagnostic tools impedes to assess individuals' susceptibility to develop ALD and the pathogenesis of this disease in humans remains poorly understood. The lack of advances in this field is mostly due to the difficulties in concluding clinical trials in patients with active alcohol intake, the absence of interest in this field from drug companies and the lack of experimental models of advanced ALD (Altamirano et al. 2011). As main consequence, since the early 1970s, when corticosteroids were proposed for the treatment of severe AH (Helman et al. 1971), few drugs for the treatment of ALD have been developed. The only effective therapy for all these patients seems to be abstinence. Abstinence is also a critical step for patients with advanced ALD who may eventually require liver transplantation, because patients

who actively consume alcohol are not eligible for most transplantation programs. For those patients who find alcohol cessation impossible, treatments for attenuate the progression of liver disease and reduce mortality rate are urgently needed.

ALD includes a large spectrum of diseases such as asymptomatic fatty liver, steatohepatitis, progressive fibrosis, end-stage cirrhosis and HCC. As early reaction to alcohol intake, about 90% of heavy drinkers develop fatty liver, which is usually asymptomatic and reversible in case of abstinence. Nevertheless, persistent heavy alcohol intake in presence of fatty liver, can lead to hepatic inflammation, mostly characterized by polymorphonuclear cells (PMN) infiltration and hepatocellular damage mainly due to reactive oxygen species (ROS) and other products of alcohol metabolism liberation. Both hepatic inflammation and hepatocellular damage are two of the hallmarks that define AH (Elphick et al. 2007). In 20-40% of cases, patients develop liver fibrosis and of these, 8-20% also develop cirrhosis, which confers a high risk of complications such as ascites, variceal bleeding, hepatic encephalopathy, renal failure, bacterial infections and development of HCC (Adachi et al. 2005; Lucey et al. 2009) (Figure 1). In patients with underlying ALD and heavy alcohol consumption may occur episodes of superimposed AH, typically in most severe cases and in patients with liver cirrhosis, AH leads to severe complications related to liver failure and portal hypertension and presents high short-term mortality (Lucey et al. 2009; Altamirano et al. 2011; Gao et al. 2011). In the last decades have been described several risk factors for ALD including: sex, obesity, drinking patterns, dietary factors, environmental factors, non-sex-linked genetic factors and cigarette smoking (Altamirano et al. 2011; Gao et al. 2011). However, the mechanisms underlying the acute phase of AH and its bad prognosis remain mostly unknown.

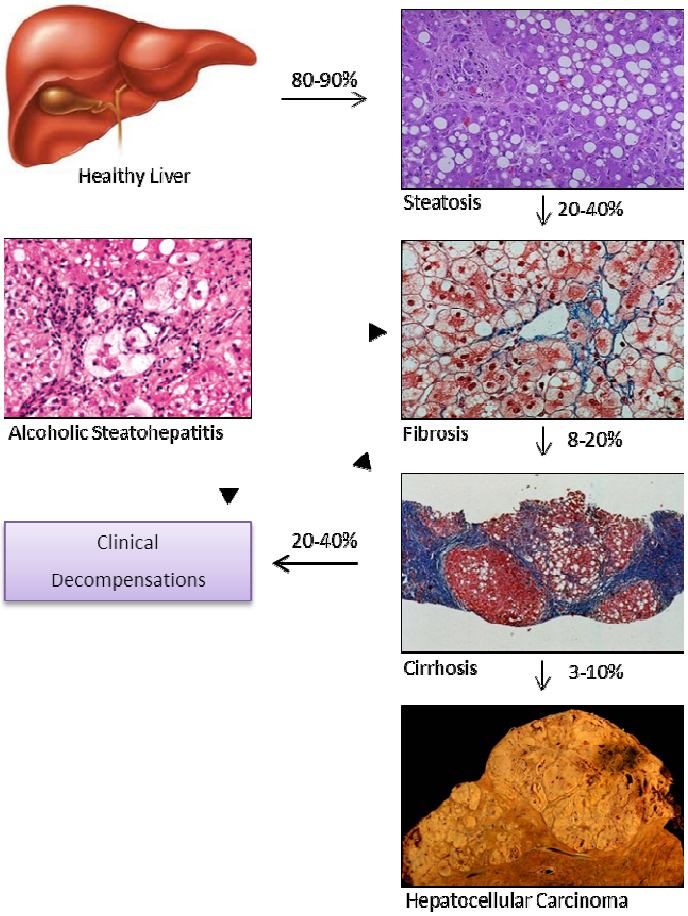


Figure 1. *Spectrum of alcoholic liver disease (Adapted from Altamirano J, Bataller R. 2011)*

1.1 Alcoholic Hepatitis

Alcoholic hepatitis is the most severe form of ALD and is a life-threatening condition. AH develops in patients with heavy alcohol intake and histologically is characterized by steatosis, profound hepatocellular damage, inflammatory infiltrate and pericellular fibrosis (Colmenero et al. 2007; Dominguez et al. 2009). The prevalence of AH has not been accurately determined but it is believed to occur in 10% to 35% of heavy drinkers and underlying severe liver disease (Gao et al. 2011). The severe forms of AH are associated with liver failure and portal hypertension, leading to a short-term poor prognosis and high short-term mortality (Dominguez et al. 2008).

AH involves several hepatocellular and inflammatory processes all interacting each other's and including: hepatocytes apoptosis, progenitor cells expansion, fibrosis and adaptive and innate immunity activation. All these features of AH will be discussed and developed within this thesis.

1.1.1 Prognosis and Management

Several prognostic models have been developed to classify patients with AH who present high risk of death within 1–3 months of their hospitalization. Maddrey's discriminant function (DF) (Maddrey et al. 1978) was the first score to be developed and remains the most commonly used. According to this score, severe AH is defined as a DF>32 and the reported 1-month survival of untreated patients with a DF>32 ranges from 50% to 65% (Carithers et al. 1989; Phillips et al. 2006). Other prognostic scores, such as the Model for End-Stage Liver Disease (MELD) and the Age, Bilirubin, INR, and Creatinine score (ABIC); have been proposed to be used for the characterization of AH (Dunn et al. 2005; Forrest et al. 2005; Srikureja et al. 2005; Dominguez et al. 2008). One of the most important limitations noticed in many of these prognostic models developed for AH, is that they only stratify patients into two categories, severe and non-severe, and only early mortality risk is considered. Indeed, a percentage of patients may not comply the criteria for severe AH and may die at time points longer than which used in the scores. For this reason, the prognostic models for AH should be better defined and should include more groups and contemplate survival times in a longer term. New advances in this direction have been done and the recently defined ABIC score classifies patients according to low, intermediate or high risk of death (Dominguez et al. 2008) and this classification allows a better evaluation of therapies for AH.

Another factor that predicts mortality in AH is the development of acute kidney injury (AKI), defined as an absolute increase of serum creatinine of 0.3 mg/dL, or a 50% increase above baseline. AKI is associated with a marked decrease in 90-day survival. Interestingly, patients with systemic inflammatory response syndrome (SIRS) at admission developed AKI in much higher proportions compared to those without SIRS. Moreover, SIRS has also been associated with decreased 90-day survival (Altamirano et al. 2012). Due to their recent discovery in correlation with AH patient's outcome, neither SIRS nor AKI have been incorporated into a prognostic model at this point, but certainly represent important events that should be taken in consideration in the development of new scores.

The histological features of AH include: centrilobular ballooning of hepatocytes, neutrophil infiltration, Mallory-Denk body hyaline inclusions, steatosis, fibrosis and eventually cirrhosis (Lucey et al. 2009; Gao et al. 2011). Recently, our group has performed a large multicentric study to develop a new histological score able to predict short-term survival in patients with AH. The resulting alcoholic hepatitis histological score (AHHS) includes 4 parameters: fibrosis stage, PMN infiltration, type of bilirubinostasis and presence of megamitochondria, which are independently

associated with patients' survival. By combining these parameters in a semi-quantitative manner, we were able to stratify patients into low, intermediate and high risk for death within 90 days (Altamirano et al, manuscript accepted for publication in Gastroenterology).

In addition to the use of corticosteroids, other strategies directed to modulate the inflammatory response in AH have been suggested and tested with limited efficacy or even with a negative impact on patients' prognosis. This is the case of tumor necrosis factor (TNF)-α blockade, which has been demonstrated to be associated with an increased susceptibility to severe bacterial infections (Naveau et al. 2004) and high mortality. Therefore currently, anti-TNF-α agents are not recommended for treatment of AH.

1.2 New Therapeutic Targets in Alcoholic Liver Disease

As animal models do not accurately mimic the main findings observed in advanced ALD in humans, translational studies using human samples are urgently needed to identify new therapeutic targets. Several epidemiological studies have identified many genes that mediate liver injury and fibrosis in ALD. However, the key genes involved in the pathogenesis of ALD remain uncertain. Although the correlation between serum levels of molecular mediators of ALD such as TNF-α and disease severity have been largely investigated, the pathogenic significance of these associations remained unclear, since increased serum levels of cytokines could be the product of impaired liver clearance or ongoing bacterial infections. Therefore, despite some of these molecules are involved in the physiopathology of ALD, others might be more useful as markers of presence of inflammation or fibrosis than for their role as mediators of the disease. A more rational approach would be to investigate the expression and/or activation of different mediators of ALD in liver samples from patients with ALD, and to correlate these findings with disease severity and the patient's outcome. The most relevant mediators for ALD identified until today using human samples include: CXC chemokines (IL-8, GRO-α) (Colmenero et al. 2007; Dominguez et al. 2009), IL-22/STAT3 pathway (Gao et al. 2011), osteopontin (Morales-Ibanez et al. 2013), gut microbiota and lipopolysaccharide (Mencin et al. 2009), endocannabinoids (Patsenker et al. 2011), and inflammasomes (Szabo et al. 2012).

2. HEPATOCELLULAR DAMAGE AND REGENERATION

Ethanol consumption has a direct effect on hepatocellular damage due to the generation of metabolites such as acetate, ROS, and acetaldehyde. Moreover, ethanol and its metabolites trigger a cascade of events, including hepatocytes apoptosis and necrosis and liver regeneration, which lead to the activation and perpetuation of hepatic inflammation, increased extracellular matrix deposition and progressive fibrosis.

2.1 Ethanol Metabolism and Hepatotoxicity

The metabolism of ethanol in the liver takes place mostly in the hepatocytes. In hepatocytes, the enzymes responsible for ethanol metabolism into acetaldehyde are alcohol dehydrogenase (ADH), cytochrome P450 2E1 (CYP2E1) and catalase. In the cytosol, ADH converts ethanol to acetaldehyde, a reaction that involves an intermediate carrier of electrons and nicotinamide adenine dinucleotide (NAD+), which is reduced by two electrons to NADH. Catalase, located in cell bodies called peroxisomes, requires hydrogen peroxide (H_2O_2) to oxidize alcohol. CYP2E1, present predominantly in the cell's microsomes, plays an important role in metabolizing ethanol to acetaldehyde in presence of important ethanol concentrations.

ROS are generated as consequence of ethanol metabolism and provoke lipid peroxidation, mitochondrial glutathione and S-adenosylmethionine depletion. All these products make hepatocytes susceptible to damage. When produced, acetaldehyde is rapidly metabolized into acetate and NADH by aldehyde dehydrogenase 2 (ALDH2) in the mitochondria (Figure 2) (Zakhari 2006). Acetaldehyde is a reactive specie extremely toxic to hepatocytes due to the formation of protein and DNA adducts, which promote glutathione depletion, lipid peroxidation and mitochondrial damage. Most of the acetate resulting from acetaldehyde, quickly escapes the liver and reaches the circulation, where it is metabolized to CO_2 in the heart, skeletal muscle, and the brain cells. Acetate is also metabolized to acetyl CoA, which is involved in lipid and cholesterol biosynthesis in the mitochondria of peripheral and brain tissues and it is hypothesized that upon chronic alcohol intake the brain starts using acetate rather than glucose as a source of energy (Zakhari 2006; Farfan Labonne et al. 2009; Setshedi et al. 2010). Moreover, the presence of acetate, increases blood flow into the liver and depresses the central nervous system, as well as affects various metabolic processes. Although acetate has no direct hepatotoxic effects, it seems that acetate can regulate the inflammatory response in patients with AH inducing the up-regulation of pro-inflammatory cytokines in macrophages (Shen et al. 2009; Kendrick et al. 2010).

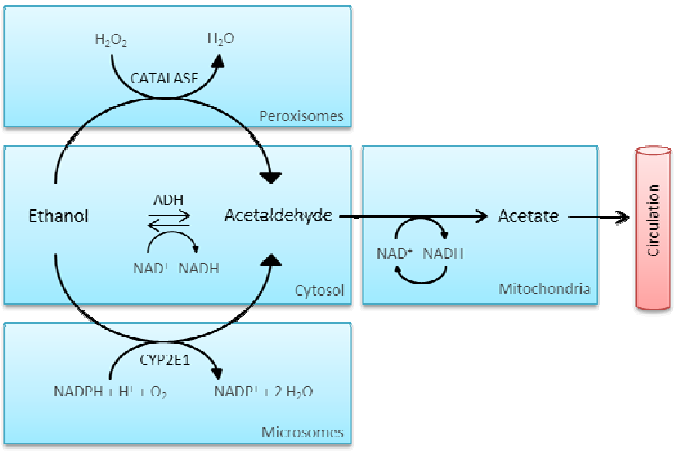


Figure 2. *Ethanol metabolism (Adapted from Zakhari S.2006)*

2.2 Hepatocyte Apoptosis

Hepatocytes apoptosis is an important pathologic feature of human ALD. Apoptosis is the result of numerous complex mechanisms, including ethanol-mediated hepatotoxicity, generation of ROS, and inhibition of survival genes and induction of pro-apoptotic signaling molecules. Although several stimuli can trigger apoptosis, two molecular pathways are the main transducers of the apoptosis signaling: the death receptor pathway (also called the extrinsic pathway) and the mitochondrial pathway (also called the intrinsic pathway). Activation of a large variety of intracellular proteases called caspases and endonucleases, is the result of both extrinsic and intrinsic pathway, and leads to the degradation of the cellular components. The extrinsic pathway initiates at the plasma membrane when a family of cytokine receptors named death receptors (such as tumor necrosis factor receptor 1 (TNF-R1), Fas/CD95, and tumor necrosis factor related apoptosis inducing ligand receptors 1 and 2 [TRAIL-R1 and TRAIL-R2]) are bound and activated by their ligands (TNF-α, Fas ligand (FasL)/CD95L, TRAIL) (Ashkenazi et al. 1998; Guicciardi et al. 2005). The cell death program is initiated by caspases, which are constitutively expressed as inactive pro-enzymes and require proteolytic processing for their activation.

Within the intracellular proteins involved in the regulation of apoptosis, it is important to stress the Bcl-2 family, which includes both pro- (Bax, BAD, Bak, Bok) and anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w) members and integrate death and survival signals (Cory et al. 2002). Fas is another

important protein in apoptosis, is widely expressed in all liver cell types, being the Fas/FasL system the pathway most commonly used by immunocytes to kill virally infected cells (Krammer 2000; Guicciardi et al. 2005). The high expression levels of death receptors in hepatocytes are the main reason why apoptosis in the liver occurs mainly via the extrinsic pathway.

Patients with AH present hepatocytes apoptosis, which directly correlates with disease severity (Natori et al. 2001); and that apoptotic hepatocytes often co-localize with infiltrating neutrophils, suggesting that apoptosis would trigger an inflammatory response (Ziol et al. 2001; Jaeschke 2002). Alcohol-induced hepatocyte apoptosis has been explained by several mechanisms, and one of the most convincing is that once ethanol induces CYP2E1, the formation of ROS and lipid peroxides, leads to hepatocytes sensitization and apoptosis (Kurose et al. 1997; Tagami et al. 2003). In fact ROS, whose production is driven by increased availability of the reduced form of NAD due to mitochondrial acetaldehyde metabolism, may cause mitochondrial dysfunction and release of pro-apoptotic factors (such as cytochrome *c*) into the cytosol where they promote caspases activation. Consistently, hepatocytes apoptosis has been shown to be reduced by antioxidants in rats exposed to acute ethanol intoxication (Kurose et al. 1997).

Some death receptors and their ligands, especially Fas/FasL, have been found strongly expressed in hepatocytes of patients with AH compared with healthy controls or patients with alcoholic liver disease without hepatitis, which could increase the sensitivity to apoptosis. Levels of circulating Fas and FasL were also found to be elevated in patients with severe AH, however, its cell source have not been well defined (Taieb et al. 1998). Because both Fas and FasL are expressed on the same cell type, it has been proposed that hepatocyte apoptosis could occur by autocrine and/or paracrine mechanisms (Taieb et al. 1998).

2.3 Liver Regeneration

In response to liver injury and/or loss of hepatic tissue, the liver can regenerate and recover its original mass mainly via proliferation of remaining adult hepatocytes, biliary epithelial cells and non-parenchymal cells (Gao et al. 2011).

Under pathogenic conditions, in which proliferation of hepatocytes is inhibited, liver progenitor cells (LPC), also known as oval cells or ductular hepatocytes, proliferate and differentiate into hepatocytes or biliary epithelial cells (Michalopoulos 2007). LPC are thought to reside in the terminal branches of the biliary tree (canals of Hering), at the interface between portal tracts and liver lobule (Theise et al. 1999). LPC proliferate and expand during severe hepatic injury or when there is an impairment of hepatocytes regeneration capacity giving rise to what is known as ductular reaction (Duncan et al. 2009). LPC are believed to contribute to hepatocytes regeneration and to participate in the recovery of liver function; however, data regarding the degree of their contribution is not conclusive (Furuyama et al. 2010; Carpentier et al. 2011). It has been recently reported that LPC proliferate in chronic liver disease and that their proliferation is particularly important in ALD. The number of hepatic progenitor cells is increased in ALD, probably due to the combination of oxidative liver damage induced by ethanol metabolism, which promotes hepatocytes apoptosis and inhibits hepatocytes proliferation; and in part because alcohol itself seems to trigger progenitor cells expansion (Jung et al. 2008; Sancho-Bru et al. 2012).

The presence of an important ductular reaction in patients with ALD (Lowes et al. 1999; Jung et al. 2008) has been confirmed in patients with AH in more recent studies from our laboratory (Sancho-Bru et al. 2012), where we showed that liver progenitor cells markers (such as Keratin-7, Prominin-1 and Epcam) are up-regulated in patients with AH and correlate with patient's outcome. However, the important LPC expansion in patients with AH does not lead to an improved hepatic function since patients with increased expression of LPC markers show higher mortality rates compared to those with less progenitor cell expansion. The important accumulation of liver progenitor cells in AH suggests that in this condition there is a defect in cell maturation to hepatocytes, and a poor contribution of LPC to the recovery of the liver function.

3. LIVER FIBROSIS

Hepatic fibrosis is the consequence of hepatic wound-healing to repeated injury, and is characterized by deregulated extracellular matrix (ECM) production and accumulation. In case of acute or limited insult, hepatic fibrosis is transient, and liver architecture can return to normality. Nevertheless, if the liver injury is constant, chronic inflammation and accumulation of ECM persist, leading to a progressive substitution of liver parenchyma by fibrotic tissue. This process results in cirrhosis, the end stage of progressive fibrosis, which is characterized by a poor outcome and high mortality rate. Patients with chronic liver injury takes about 20 to 40 years in developing cirrhosis and this process is also affected by both genetic and environmental factors.

The liver is composed by parenchymal cells (hepatocytes), resident non-parenchymal cells, including HSCs, endothelial cells; Kupffer cells (KCs) and cholangiocytes. The sinusoid is the hepatic microvascular unit characterized by discontinuous and fenestrated endothelium. It receives blood from terminal branches of the hepatic artery and portal vein at the edge of hepatic lobules and delivers it into central veins. Hepatocytes are separated from the sinusoids by the sub-endothelial space of Disse, where HSCs reside. Since endothelial cells have no basement membrane, they are separated from hepatocytes only by the space of Disse, which contains a low-density matrix that is essential for maintaining the differentiated function of parenchymal cells and is sufficiently porous to allow metabolic exchange between the blood and hepatocytes (Figure 3).

The main causes of liver fibrosis include chronic HCV infection, alcohol abuse, and nonalcoholic steatohepatitis (NASH). As said before, the accumulation of ECM proteins may cause an alteration in the architecture of the liver by forming a fibrous scar, and the subsequent development of nodules of regenerating hepatocytes, which altogether may lead to cirrhosis. Cirrhosis produces hepatocellular dysfunction and increased intrahepatic resistance to blood flow, which result in hepatic insufficiency and portal hypertension, respectively (Gines et al. 2004) and is associated with an increased risk of HCC (Bataller et al. 2005). Esophageal variceal bleeding, ascites and/or hepatic encephalopathy are common decompensations of end stage liver diseases and cirrhosis, which are associated with bad prognosis (Bataller et al. 2005).

In response to acute liver injury such as viral hepatitis, parenchymal cells regenerate and replace apoptotic or necrotic cells, a process that is associated with an inflammatory reaction and limited deposition of ECM. Nevertheless, if the hepatic injury persists, the liver regeneration fails, and hepatocytes are substituted with abundant ECM, including fibrillar collagen. Specifically,

following chronic liver injury, the regenerative capacity of hepatocytes is reduced and they undergo apoptosis; inflammatory lymphocytes infiltrate the hepatic parenchyma, and KCs activate and produce inflammatory and fibrogenic mediators. HSCs, the main fibrogenic cell type of the liver, proliferate and undergo a phenotypic activation, secreting large amounts of ECM proteins. As consequence of all these changes, sinusoidal endothelial cells lose their fenestrations, and the tonic contraction of HSCs provokes increased resistance to blood flow in the hepatic sinusoid (Bataller et al. 2005).

In normal liver, ECM is a dynamic substrate with a precisely regulated balance between synthesis and degradation. During chronic liver injury, this balance is broken and ECM production exceeds ECM degradation leading to hepatic fibrosis. During hepatic fibrogenesis, the changes in ECM not only affect its quantity but also the quality of the matrix (Hernandez-Gea et al. 2010). The most important structural components of ECM in liver are: collagen, proteoglycans, laminin, fibronectin, and matricellular proteins. As a consequence of liver injury normal liver matrix is replaced with a new fibrillar matrix, mainly composed of collagens I and III and fibronectin (Hernandez-Gea et al. 2010). These quantitative and qualitative changes in ECM arrangement (termed capillarization) (Benyon et al. 2000) alter the matrix microenvironment and create a functional and physical barrier to the bidirectional flow of plasma that occurs between sinusoidal lumen and hepatocytes, leading to altered hepatic function. However, it is important to underline that the accumulation of ECM during liver fibrosis is the result of both increased synthesis and decreased degradation (Arthur 2000). The decreased activity of ECM-removing metallo-proteases (MMPs) during fibrosis processes is mainly due to an increase of metallo-proteases specific inhibitors (TIMPs).

The distribution of the fibrotic tissue in the liver depends on the type of liver injury. In chronic viral hepatitis and chronic cholestatic disorders, the fibrotic tissue is located around portal tracts, whereas in alcohol-induced liver disease it locates mainly in pericentral and perisinusoidal areas (Pinzani 1999; Bataller et al. 2005; Altamirano et al. 2011).

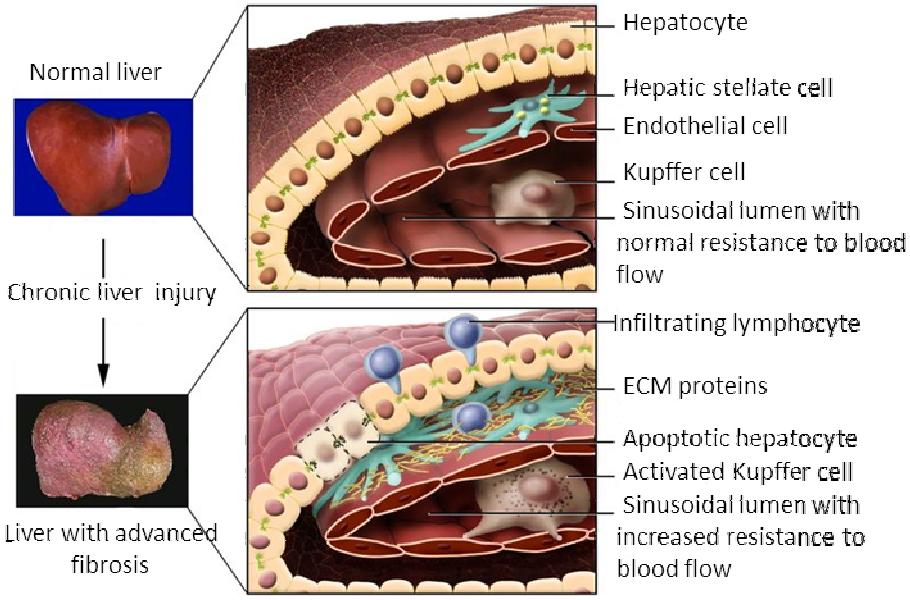


Figure 3. *Changes in the hepatic architecture associated with advanced hepatic fibrosis (From Bataller, R. and D. A. Brenner 2005)*

As mentioned, HSCs are the main ECM-producing cells in the injured liver (Gabele et al. 2003) and their specific role in liver fibrogenesis will be discuss in detail in next paragraph. Importantly, in addition to HSCs, other hepatic cell types also have fibrogenic potential. In fact, myofibroblasts derived from small portal vessels can proliferate around biliary tracts in cholestasis-induced liver fibrosis to initiate collagen deposition. The relative importance of each cell type in liver fibrogenesis may depend on the type of liver injury. While HSCs are the main fibrogenic cell type in pericentral areas, portal myofibroblasts may prevail when liver injury occurs around portal tracts (Kinnman et al. 2002; Bataller et al. 2005). It is also important to stress that during fibrogenesis, different hepatic cell types interplay between them promoting and perpetuating hepatic inflammation and progression (Kmiec 2001) (Figure 4).

Indeed, during hepatic fibrogenesis it takes place a complex interplay between different hepatic cell types including platelets, hepatocytes, inflammatory cells, sinusoidal endothelial cells and KCs. Platelets are the first cells recruited to the site of injury to limit blood loss by forming aggregates converting fibrinogen into fibrin. Platelets display many properties which are relevant in wound healing as the initiation of coagulation; and are also capable to release important growth factors during fibrogenesis such as platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β), which are strong stimulators of HSCs (Henderson et al. 2007). Hepatocytes, targets for several hepatotoxic agents (Higuchi et al. 2003), when damaged release ROS and fibrogenic

mediators to induce recruitment of white blood cells by inflammatory cells. Moreover, apoptosis of damaged hepatocytes, stimulates the fibrogenic action of HSCs (Canbay et al. 2004). Inflammatory cells, either lymphocytes or neutrophils, stimulate HSCs to secrete collagen (Casini et al. 1997) and once activated, HSCs secrete inflammatory chemokines, express cell adhesion molecules and can modulate the activation of lymphocytes (Vinas et al. 2003). Moreover, different T helper cells influence fibrosis progression, being Th2 the more active in fibrogenesis and mainly responsible for the production of TGF-β (Shi et al. 1997). Therefore, occurs a vicious circle where inflammatory and fibrogenic cells stimulate each other and perpetuate the process of liver damage and repair (Maher 2001).

KCs, hepatic resident macrophages, play a major role in liver inflammation by releasing ROS and cytokines and are almost always found in close proximity to collagen-producing myofibroblasts (Wynn et al. 2010). During fibrosis progression, macrophages are likely to stimulate HSCs activation via release of paracrine mediators including TGF-β1 and PDGF (Wahl et al. 1990; Bonner et al. 1991); moreover, recent studies revealed a novel function of macrophages promoting HSCs and myofibroblasts survival (Pradere et al. 2013). However, the simultaneous loss of macrophages and HSCs during fibrosis recovery suggests that macrophages may also have a counterbalance effect on HSCs promoting HSCs apoptosis by the expression of TRAIL and other pro-apoptotic stimuli (Bataller et al. 2005; Henderson et al. 2007). Therefore, macrophages play a pivotal role in fibrosis processes inducing both liver injury and repair synchronously. As described in literature, depletion of macrophages when liver fibrosis is advanced results in reduced scarring and fewer myofibroblasts and by contrast, macrophage depletion during recovery leads to a failure of matrix degradation (Bataller et al. 2005; Henderson et al. 2007). These data suggest that exist functionally distinct populations of macrophages with opposite but complementary functions in the same tissue, and that these macrophages play critical roles in both injury and recovery phases of fibrogenesis (Bataller et al. 2005; Henderson et al. 2007).

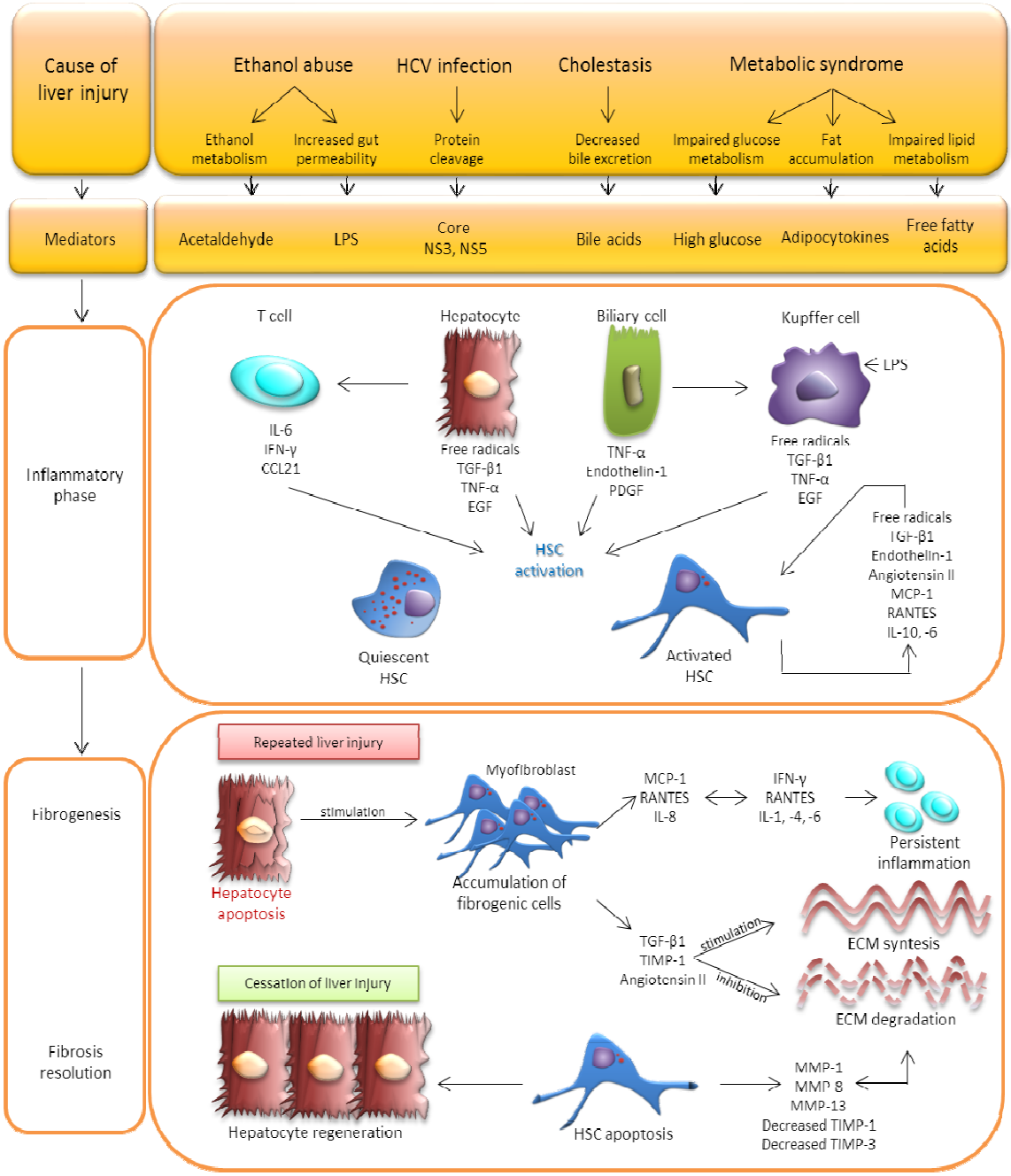


Figure 4. *Pathogenesis of hepatic fibrosis (Adapted from Bataller, R. and D. A. Brenner 2005)*

3.1 Hepatic stellate cells

Hepatic stellate cells (from the latin “stella”, that means star) were first described in 1876 by Von Kupffer. In normal liver, HSCs are approximately the 1.4% of total liver cells and about the 30% of no-parenchymal hepatic cells. As previously mentioned, HSCs are located in the hepatic sinusoid in the perisinusoidal space of Disse, between endothelial cells and hepatocytes. In its quiescent state, HSCs show large perinuclear lipid droplets, which serve as main storage site for retinol (vitamin A) and are crucial in the regulation of the retinoic acid homeostasis in healthy liver (Carpino et al. 2004).

As a result of liver injury, HSCs undergo activation and turn from a quiescent, vitamin A-storing cell type, to an activated myofibroblast-like cell type. The presence of pro-inflammatory (such as MCP-1) and pro-fibrogenic (such as TGF-β and PDGF) mediators in damaged liver induce several new phenotype features in activated HSCs. HSCs activation is characterized by an important change in gene expression pattern that to some extend is regulated by epigenetic marks and also by micro RNA (Mann et al. 2008; Mann et al. 2009; Mannaerts et al. 2010; Perugorria et al. 2012; Yang et al. 2012; Szabo et al. 2013). Once activated, HSCs show increased cell migration and adhesion, expression of α-smooth muscle actin (α-SMA), increased proliferation, production of chemotactic molecules capable to recruit inflammatory cells to the site of the injury, contractility, loss of vitamin A-storage capacity, increased rough endoplasmic reticulum, changes in cytoskeletal organization and cellular morphology and acquisition of fibrogenic capacity (Milani et al. 1990; Bataller et al. 2005; Shafiei et al. 2006) (Figure 5).

Activation of HSCs includes two main stages: the initiation and the perpetuation stage. During the initiation phase, HSCs become more responsive to proliferative and fibrogenic cytokines by up-regulation of membrane receptors and undergoing the initial changes toward a myofibroblast-like cell. Once HSCs have been induced to up-regulate their cytokine receptors, enhanced cell proliferation and fibrogenesis will be perpetuated by the continue release of mediators from chronically damages tissue (Moreira 2007). Once activated, HSCs also provoke an up-regulation of hepatic expression of ECM genes, matrix-degrading enzymes and their respective inhibitors, resulting in matrix remodeling and accumulation in the injured liver (Knittel et al. 1999; Bataller et al. 2005).

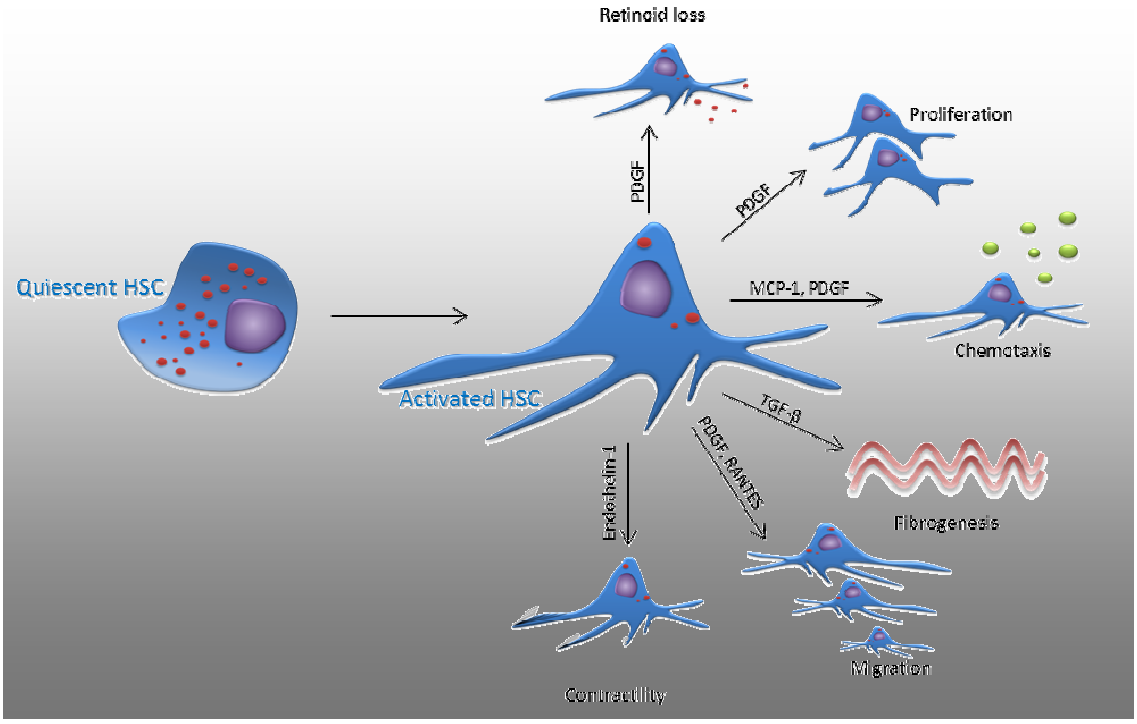


Figure 5. *Phenotypic features of hepatic stellate cell activation during liver injury (Adapted from Friedman 2002)*

As said before, a complex cross talk between different hepatic cell types occurs during hepatic fibrogenesis, which includes platelets, hepatocytes, inflammatory cells, sinusoidal endothelial cells and KCs, all interacting with HSCs. Each of the hepatic cell type interacting with HSCs release a subset of mediators, which will induce different effects on them and on liver fibrosis. PDGF and TGF-β are the two best characterized cytokines which participate in HSCs activation, being PDGF the main mediator of HSCs proliferation and TGF-β the most important cytokine stimulating fibrogenesis in HSCs (George et al. 1999; Borkham-Kamphorst et al. 2004; Bataller et al. 2005; Gressner et al. 2006). Other mediators of HSCs activation have been described to directly stimulate fibrogenesis such as MCP-1, endothelin 1, angiotensin II and adipokines such as leptin (Moreira 2007) and ghrelin (Moreno et al. 2010). Moreover, several molecules including TNF-α, TGF-β, TIMP-1, collagen-1 and integrins have been shown to have fibrogenic properties by causing inhibition of HSCs apoptosis, thereby contributing to the increased number of HSCs in damaged liver (George et al. 1999).

3.2 Molecular Drivers of Liver Fibrosis

Several factors can regulate hepatic fibrosis, and a multitude of mediators have been described to participate in liver fibrogenesis, including hormones, bacterial products, adipokines and a complex network of pro-fibrogenic cytokines (Bataller et al. 2005; Friedman 2008).

Among hormones, prostaglandin E2 (PGE_2) belonging to the hormone class of the eicosanoids and mainly produced by activated Kupffer cells, is a potent physiological suppressor of liver fibrosis. PGE_2 induce cAMP production and protein kinase activation to inhibit MAPK signaling and resulting in a hepatoprotective function (Lotersztajn et al. 2005). Fatty acids and other agonists activate peroxisome proliferator–activated receptors (PPR) have also been described to participate in liver fibrogenesis (Chen et al. 2013). As well described in literature, bacterial products such as LPS are also important players in both hepatic inflammation and fibrosis by binding and activating TLR4 and stimulating Myeloid differentiation primary response gene (88) (MyD88)-depentent pathway to induce fibrogenic signals.

Persistent inflammation almost always precedes hepatic fibrosis; therefore, it makes sense that inflammatory cytokines play a key role in fibrosis. Indeed, following liver injury, several cell types including KCs, hepatocytes, HSCs, natural killer (NK) cells, lymphocytes, and dendritic cells (DC), can secrete inflammatory cytokines. Cytokines family includes chemokines, interferons, interleukins, growth factors, soluble neurohumoral ligands (endocannabinoids) and adipokines (Hernandez-Gea et al. 2010). Adiponectin and leptin are the two main adipokines implicated in liver injury. Leptin can mediate its biological effects through one of several leptin receptors (ObRa to ObRf) via activation of the Janus kinase 2 (JAK2) and signal transducer and activator of transcription 3 (STAT3) pathways (Marra et al. 2009) (Figure 6). Leptin has pro-fibrogenic properties and also plays a role in promoting the proliferation, migration, and metastasis of hepatocellular carcinoma and cholangiocarcinoma cells (Marra 2007; Saxena et al. 2007; Fava et al. 2008; Hernandez-Gea et al. 2010). Moreover, several cytokins including IFN-γ, has been described to activate the JAK-STAT signaling pathway. When cytokines bind to their receptors activate receptor-associated tyrosine kinases (JAK1, JAK2, JAK3,Tyk2), which interact with the STAT proteins (STAT1 to 6) (Gao 2005; Moreno et al. 2008). STAT1 has been proposed to negatively regulate liver fibrosis through several mechanisms, including inhibition of HSCs proliferation, suppression of PDGF receptor (PDGFR)-β expression, inhibition of TGF-β/Smad3 signaling, and stimulation of NK cell cytotoxicity (Jeong et al. 2006). Adiponectin has been described to inhibit hepatic fibrogenesis both in vitro and in vivo (Marra et al. 2009). Following adiponectin binding to the specific receptors AdipoR1 and AdipoR2

in HSCs, downstream effects are mediated by adenosine monophosphate (AMP)–activated protein kinase (AMPK) and peroxisome proliferator–activated receptor α (PPAR-α) (Adachi et al. 2005). AMPK activation produces ATP and impedes processes that consume ATP, apart from those crucial for short-time survival. Furthermore, in our group, we recently discovered the role of a new adipokine, ghrelin, which attenuates hepatocellular injury and signaling during fibrogenesis (Moreno et al. 2010).

Growth factors are also members of the cytokine family. As mentioned before, the most important growth factors involved in HSCs activation and collagen syntheses are PDGF and TGF-β. PDGF is a dimeric protein that signal via the tyrosine kinase receptors PDGFR-α and PDGFR-β and all PDGF isoforms have been found up-regulated during HSCs activation and correlates with degree of hepatic inflammation and fibrosis (Moreno et al. 2008). PDGFRs in part activate phosphoinositol 3-kinase (PI3K)/Akt, which also transduces signals for other tyrosine kinases (e.g., vascular endothelial growth factor -VEGF), cytokine receptors, integrins, adipokines (leptin), and G protein–coupled receptor stimulators (e.g. angiotensin II and thrombin). When a tyrosine kinase receptor binds to its receptor, its tyrosine residues become autophosphorylated, and this provokes the recruitment of PI3K to the membrane. Once activated and placed to the membrane, PI3K phosphorylates phosphoinositol lipids, which translocate Akt to the plasma membrane. After its recruitment, Akt is phosphorylated by phosphoinositide-dependent kinase and thereby activated and regulate several cell functions (Paez et al. 2003). Growth factor receptors may also signal through MAPK signaling pathways. The MAPK family includes extracellular signal–regulated kinase, c-Jun N-terminal kinase (JNK), and p38 MAPK. Chemokines and proliferative peptides such as PDGF, thrombin, angiotensin II, VEGF, leptin; activate these molecules. Once activated, they recruit the signaling molecule RAS, which leads to the transcription of proliferative and pro-fibrogenic molecules (Bonacchi et al. 2001; Pinzani 2002; Bataller et al. 2003).

Several cell types secrete TGF-β, which has been described to be present in three major isoforms (TGF-β1, TGF-β2, and TGF-β3). Between them, TGF-β1 is the principal isoform implicated in liver fibrosis, principally produced by monocytes and macrophages. TGF-β1 is stored as an inactivated protein and once activated, signals via its receptors to Smad proteins, which improve the transcription of target genes, including procollagen I and procollagen III (Inagaki et al. 2007). When TGF-β1 binds to type II receptor, receptor II dimerizes with receptor I and the resulting heterodimer can then translocate into the nucleus and regulate transcription (Inagaki et al. 2007).

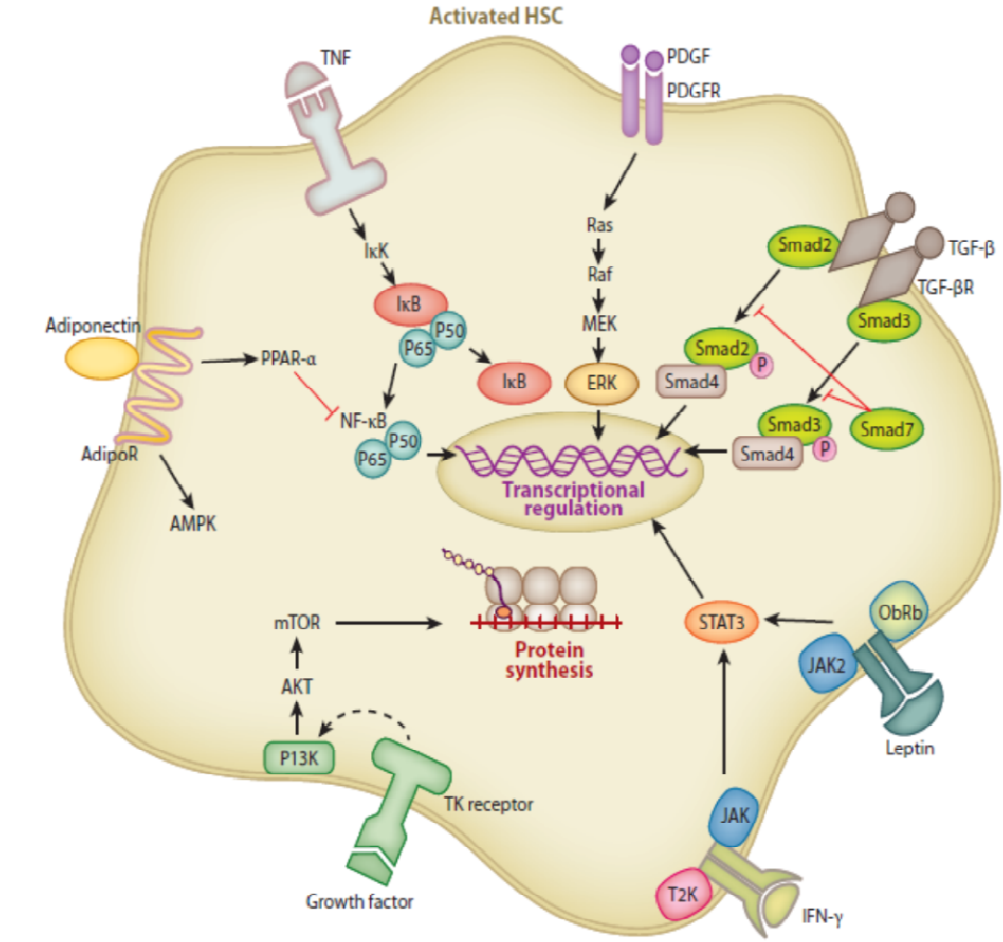


Figure 6. *Main cytokine signaling pathways in activated HSCs*

(From Hernandez-Gea 2010)

3.3 Liver Fibrosis in Alcoholic Liver Disease

Chronic alcohol consumption is one of the major causes of liver fibrosis which, as described before, is characterized by excessive accumulation of ECM proteins due to an impaired ECM degradation and production (Purohit et al. 2006). Although the underlying mechanisms of alcoholic liver fibrosis are not yet fully understood, several studies provide potential new mechanisms that will have to be investigated. First of all, acetaldehyde and ROS generated by hepatic alcohol metabolism trigger collagen and TGF-β production in HSCs through a paracrine mechanism (Nieto et al. 2002; Svegliati-Baroni et al. 2005). Secondly, hepatocytes apoptotic bodies induced by alcohol are phagocytized by KCs and HSCs, resulting in the production of TGF-β1 and subsequently activation of HSCs (Bataller et al. 2005). Moreover, alcohol-mediated activation of KCs, such as by activation of the LPS/TLR4 signaling, also induce activation of HSCs by secreting cytokines, chemokines and ROS (Paik et

al. 2003; Schwabe et al. 2006). Moreover, NADPH oxidase-mediated ROS production also has been described to contribute to liver fibrosis (De Minicis et al. 2010). Furthermore, TLR4/MyD88 signaling in HSCs enhances TGF-β signaling, inducing liver fibrosis by down-regulating the transmembrane TGF-β receptor inhibitor named bone morphogenic protein and activin membrane-bound inhibitor (Bambi) (De Minicis et al. 2010).

Recent studies have proposed that chronic alcohol consumption predispose NK and NK T cells (NKT) to reduce their function, and this would accelerate the development of liver fibrosis (Jeong et al. 2006). As will be discuss later, NK cells have been reported to have anti-fibrotic effects via several mechanisms including kill of activated HSCs by inducing TNF-related apoptosis and via producing IFN-γ. IFN-γ, can induce HSCs cell cycle arrest and apoptosis in a STAT-dependent way and provoke autocrine activation of NK cells (Baroni et al. 1996; Jeong et al. 2006). Likewise to NK, NKT cells can also repress HSCs activation via direct killing and IFN-γ production but only in the initial phase of liver fibrosis (Park et al. 2009). Studies in humans and in animal models with alcoholic liver fibrosis show that chronic alcohol intake accelerates liver fibrosis by suppressing NK cells activity (Laso et al. 1997; Jeong et al. 2008); nevertheless further studies are indispensable to investigate the effects of alcohol on NK and NKT cells.

As previously mentioned, the deposition of ECM starts in the perisinusoidal space of Disse with a perivenular distribution, later developing to fibrosis around lobular hepatocytes, in a “chicken-wire” pattern (Moreira 2007). On a molecular level, the different morphologic hallmarks of fibrosis, related with various etiologies are probably associated to the distribution of the primary sites of HSCs activation. In ALD, the primary perivenular distribution of ethanol-induced fibrosis is thought to be linked to the mostly centrolobular expression of cytochrome P450 2E1, which participates in ethanol oxidation. As largely described, alcohol metabolism by P450 enzymes is associated with production of mediators such as ROS, which contribute to HSCs activation and induce fibrogenesis at sites of more intense enzymatic activity (Forkert et al. 1991; Takahashi et al. 1993). On the other hand, for most others chronic liver etiologies such as viral hepatitis and chronic cholestatic diseases, the inflammatory response and HSCs activation take place predominantly within and around the portal tracts, where fibrosis usually initiates (Bataller et al. 2005).

3.4 Resolution of Liver Fibrosis

Since there are many evidences in both human liver disease and animal models that hepatic fibrosis is a potentially reversible condition, resolution of hepatic fibrosis is an emerging field in hepatology. Tissue fibrogenesis was long believed to be inexorably progressive, but emerging data indicates that even in advanced disease, fibrosis is potentially reversible giving new potential insights for the treatment of this condition. Studies in several chronic liver disease caused by alcohol, autoimmune disease, biliary obstruction, hereditary hemochromatosis and non-alcoholic fatty liver disease (NAFLD), confirmed that human liver fibrosis is potential reversible (Iredale 2007). Moreover, due to the broad variety of etiologies in which fibrosis resolution occurs, it seems that the mechanisms playing a role during resolution process are generic rather than disease-specific (Ramachandran et al. 2012).

As mentioned before, fibrosis reversibility has been reported both in humans and in rodent models showing a spontaneous recovery from liver fibrosis and cirrhosis. These findings allowed the investigation that is needed to identify the critical points of this key process (Iredale et al. 1998; Murphy et al. 2002; Issa et al. 2004). It has been shown that the loss of scar-producing myofibroblasts is not sufficient for an adequate fibrosis resolution, and that the degradation of the ECM is a prerequisite. Moreover, it has been described that HSCs, myofibroblasts, KCs and other inflammatory cells involved in the fibrotic process, are also involved in the resolution process by secreting a repertoire of matrix-degrading MMPs (Benyon et al. 2001), a group of endopeptidases capable of degrading a variety of ECM constituents.

Studies in human and in experimental animal models revealed that in the fibrotic liver there is an increased number of MMPs, with a broad spectrum of activity (Iredale 2007; Ramachandran et al. 2012). In fact, even in fibrotic liver, MMPs degrade matrix but this process is attenuated by TIMPs, potent inhibitors of MMP activity *in vivo*. During activation and preceding collagen expression, hepatic myofibroblasts show a marked up-regulation of TIMP-1 and strongly inhibited MMP activity (Iredale et al. 1992; Iredale et al. 1996). In addition, elevated levels of TIMP-1 have been detected during progressive fibrosis in humans and experimental models (Benyon et al. 1996; Iredale et al. 1996). During fibrosis resolution, there is a quick reduction in TIMP levels, causing an imbalance of MMP–TIMP and resulting in increased matrix degrading activity and clear degradation of scar tissue (Issa et al. 2001; Issa et al. 2004). Therefore, TIMPs production and consequent MMPs inhibition is a key regulator in the progression and resolution of hepatic fibrosis.

A key event that occurs during resolution of fibrosis is HSCs apoptosis (Iredale et al. 1998; Murphy et al. 2002). During liver injury, when HSCs are activated in the typical wound-healing process, apoptosis is prevented, probably by signals from soluble factors and by changes in the surrounding matrix. When the damaging agent is eliminated and matrix remodeling is required, the loss of these survival factors promotes apoptosis of the activated HSCs, which enables the remodeling process by removing a key cellular source of collagen and TIMP (Henderson et al. 2007). Therefore, manipulating matrix degradation or increasing HSCs apoptosis, might be expected to decrease fibrosis and promote a return to normal liver architecture and function.

Finally, macrophages have been described to play an important role both in progression and in resolution of liver fibrosis. A number of experimental studies show that macrophages are implicated in both hepatic inflammation and fibrosis promotion (Wynn et al. 2010). Remarkably, macrophages have been described to be positioned in close proximity to activated hepatic myofibroblasts during fibrogenesis and to produce factors such as TGF-β, IL-1β, PDGF and MCP-1, which can enhance pro-fibrogenic feature of the myofibroblasts by promoting activation, proliferation, chemotaxis and survival (Friedman 2008; Wynn et al. 2010). Recent data also show the implication of macrophages as central mediators of fibrosis resolution. Interestingly, it has been shown that macrophages depletion during the resolution phase following chronic CCl_4 administration caused a failure to degrade the hepatic scar, the opposite effect to that seen with depletion during fibrogenesis (Duffield et al. 2005).

Since macrophages have been described to be involved in both hepatic fibrogenesis and resolution of fibrosis, the mechanisms supporting macrophage-mediated fibrosis resolution are estimated to be multi-factorial. Macrophages can produce a range of MMPs including macrophage-derived MMP-13, the major rodent collagenase, which has been shown to be crucial for degrading the hepatic scar (Fallowfield et al. 2007). Moreover, macrophages are also able to produce molecules such as TRAIL and MMP-9 which can promote myofibroblasts apoptosis (Elsharkawy et al. 2005; Ramachandran et al. 2012) however, *in vivo* studies demonstrating that this is a relevant mechanism in fibrosis resolution are still lacking. How can one cell type have such divergent functional effects? While this question remains incompletely answered, it is probable that heterogeneity in macrophage populations would represent a critical stage in hepatic fibrosis progression and resolution (Elsharkawy et al. 2005; Ramachandran et al. 2012). It is well documented that macrophages can assume distinct functional characteristics depending on the stimuli to which they are exposed (Mosser et al. 2008) and is possible that a specific macrophage phenotype dominate during fibrosis resolution, and that is distinct from the phenotype which

promotes fibrogenesis (Karlmark et al. 2009). For that, studies aimed at characterizing the macrophage population responsible for fibrosis resolution are needed to provide novel mechanistic insights for this process. Specifically, determining if the same macrophage population switches from a pro-fibrotic to pro-resolution phenotype *in situ* and identifying the factors mediating this switch, may facilitate the development of novel therapies designed to promote this switch and promote fibrosis resolution (Elsharkawy et al. 2005; Ramachandran et al. 2012).

4. INFLAMMATION IN ALCOHOLIC LIVER DISEASE

The activation of adaptive and innate immunity represents two of the most important events in the pathogenesis of ALD. Together with complement activation, immunity plays a key role by secreting pro-inflammatory and pro-fibrogenic cytokines typically present in the milieu of ALD, as will be discussed in detail in the next paragraphs.

4.1 Adaptive Immunity

The implication of immunity during chronic inflammation in ALD has emerged from clinical and experimental evidence. The recruitment and the activation of lymphocytes in the inflammatory infiltrate of ALD received further support by the recent demonstration of a role of Th17 lymphocytes in alcoholic hepatitis (Albano et al. 2009). Patients with AH present increased levels of circulating antibodies against lipid peroxidation adducts, and increased number of T cells in the liver, indicating that the activation of adaptive immunity is involved in the pathogenesis of ALD and specifically in AH.

T-lymphocytes have been described to be part of the inflammatory infiltrate in AH and active alcoholic-cirrhosis (Chedid et al. 1993; Dominguez et al. 2009). In either alcohol abusers and chronic alcohol-treated mice, infiltrating hepatic T cells express an activation/memory phenotype and respond to T-cell receptor stimulation by producing Th-1 cytokines such as interferon-γ (IFN-γ) and TNF-α (Song et al. 2001; Song et al. 2002). A Th-1 cytokine pattern is also evident in T cells of peripheral blood from active drinkers (Laso et al. 2005). As mentioned before, long-term alcohol intake enhances oxidative stress, which causes the generation of lipid peroxidation products. These products can induce the production of proteins adducts, that serve as antigens to activate the adaptive immune response. However, the mechanisms by which adaptive immune responses induce hepatocellular damage and inflammation in patients with AH remain unknown (Albano et al. 2009; Thiele et al. 2010; Albano 2012). The presence of circulating antibodies targeting alcohol-

altered autologous hepatocytes in patients with ALD and the hyper-production of polyclonal gamma globulins are also frequent in alcohol abusers, in association with tissues deposition of IgA (Laskin et al. 1990; Paronetto 1993). Moreover, ALD patients often show increased titers of circulating antibodies directed against non-organ-specific and liver-specific auto-antigens (McFarlane 2000). In particular, anti-phospholipid antibodies can be observed in up to 80% of patients with AH or cirrhosis (Chedid et al. 1994).

A further evidence supporting the implication of adaptive immunity in ALD comes from the recent demonstration that IL-17-producing T helper (Th-17) lymphocytes are present in hepatic inflammatory infiltrates of patients with AH and cirrhosis, in concomitance with an increase in IL-17 plasma levels (Lemmers et al. 2009; Hammerich et al. 2011). The implication of Th-17 cells in ALD is mainly important considering the growing importance attributed to these cells in the pathogenesis of several chronic inflammatory diseases including viral hepatitis B and C and primary biliary cirrhosis (Lemmers et al. 2009; Hammerich et al. 2011).

4.1.1 Cell Types in Adaptive Immune System

The main cells of the adaptive immune system are lymphocytes – B cells and T cells. B cells derived from the bone marrow and become the cells that produce antibodies and take part to the humoral immunity. In contrast, T cells mature in the thymus and differentiate into cells that either participate in lymphocyte maturation, or kill virus-infected cells taking part to the cell-mediated immunity. Cell-mediated immunity involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes and the release of several cytokines in response to an antigen. For this reason, and because its involvement in liver disease, in this thesis we will investigate in more detail T cells.

$CD4^+$ T-helper cells are major players in adaptive immunity, they help antigen-presenting cells (APCs) and $CD8^+$ cytotoxic T lymphocytes to start and promote adaptive immune responses. Activation of $CD4^+$ T cells is critical for the removal of several invading pathogens, but they can also be responsive to self-antigens, thus leading to autoimmune diseases. In order to prevent an autoimmune response, the differentiation and activation of $CD4^+$ T-helper cells has to be controlled. Nowadays, $CD4^+$ T-helper cells are divided into four major subsets, based on their expression profile of transcription factors and secreted cytokines: Th1, Th2, regulatory T cells (Treg) and Th17 (Figure 7).

Th1 and Th2 were identified in the 1980s, when it became clear that $CD4^+$ T cells could develop into independent subsets (Mosmann et al. 1986). Th1 cells are characterized by the secretion of IFNγ, a pro-inflammatory cytokine which is necessary for the activation of macrophages and involved in immunity against intracellular pathogens (Glimcher et al. 2000; Murphy et al. 2002), and Th1 cells have also been linked to cell-mediated autoimmune diseases. Th2 cells produce mainly IL-4, IL-5, and IL-13 and play an important role in allergy as well as in the clearance of various extracellular pathogens and parasites (Glimcher et al. 2000; Murphy et al. 2002; Hammerich et al. 2011).

Treg are a unique subset of $CD4^+$ T-helper cells that control effector T-cell responses to avoid autoimmune reactions. Activated Treg produce the anti-inflammatory cytokines IL-10 and TGF-β, thus suppressing the development of immune reactions (Hammerich et al. 2011). The differentiation of Treg is induced by TGF-β (Chen et al. 2003; Li et al. 2006) and is inhibited in the presence of pro-inflammatory cytokines. Treg cells are characterized by the expression on their surface of CD25 and of the transcription factors Foxp3 and STAT5.

Th17 cells are a more recently discovered subset of $CD4^+$ T-helper cells, characterized by the production of the cytokine IL-17. They represent a subtype of pro-inflammatory T-helper cells that differs from Th1 and Th2 cells in development and function. The differentiation of Th17 cells needs the combined actions of TGF-β, IL-6, and IL-21 in mice (Veldhoen et al. 2006; Wilson et al. 2007), and TGF-β, IL-23 or IL-1β in humans. These cytokines induce the expression of the orphan nuclear receptor RORγt (in mice) or RORc (in humans) (Acosta-Rodriguez et al. 2007; Volpe et al. 2008). RORγt (RORc) is necessary and sufficient for the development of Th17 cells (Ivanov et al. 2006), but the transcription factors RORα and STAT3 are also activated (Hammerich et al. 2011). Development of Th17 cells is suppressed by IFNγ and IL-4 that promote Th1 or Th2 cells, respectively (Mangan et al. 2006). TGF-β alone, in absence of other pro-inflammatory cytokines like IL-6, induces $FoxP3^+$ Treg cells instead of Th17 cells, which shows the close association between Th17 and Treg. IL-1β and IL-6 can enhance the development and expansion of human Th17. Once activated, Th17 cells secrete IL-17A, IL-17F, IL-21, IL-22, and TNF-α, which promote tissue inflammation by induction of other pro-inflammatory mediators and recruitment of leukocytes, mainly neutrophils, to the site of inflammation (Dong 2008; Ouyang et al. 2008).

Gammadelta (γδ) T cells carry antigen T-cell receptors (TCR) and are important players in the cross-talk between adaptive and innate immunity. In humans and other primates, γδ T cells represent a small percentage among peripheral blood lymphocytes (1–5%) and are a special case of

specialized and independent CD3+ T cells (Meraviglia et al. 2011). Since their discovery, γδ T cells have been shown to play a substantial role against pathogens and tumors and they were positioned in the innate immunity as cells of immune-surveillance. Within the multitude of activation signals of human γδ T cells, an important pathway is represented by toll-like receptors (TLRs). In fact, it has been shown that γδ T cells express TLR3 mRNA, thus opening the possibility that γδ T cells might respond directly to TLR3 ligands in the absence of antigen presenting cells (Wesch et al. 2006). Finally, a study published this summer in Hepatology provide some evidences about how γδ T would protect the liver from excessive inflammation and fibrosis by inhibiting HSCs (Hammerich et al. 2013).

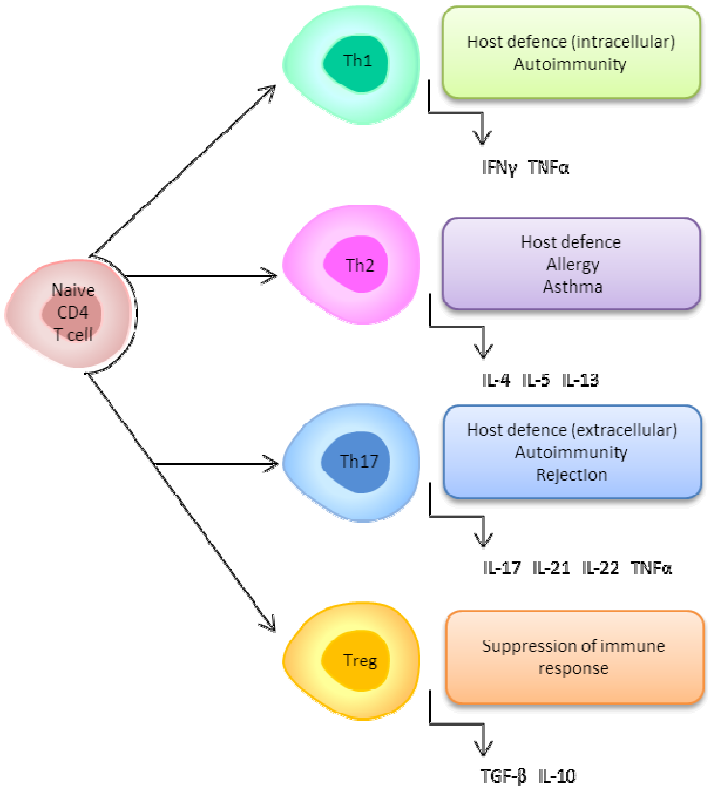


Figure 7. *Differentiation of CD4$^+$ T-cell subsets in mice (Adapted from Hammerich, et al. 2011)*

4.2 Innate Immunity

The constitutively present innate immunity represents the first line of host defense against invading organisms. Innate immunity consists of anatomic barriers (such as skin, epidermis, dermis, and mucous membranes), physiological barriers (e.g., temperature, low pH, oxygen), humoral factors (e.g., pepsin, lysozyme, antimicrobial substances, interferon, complement), phagocytic cells (e.g., neutrophils and macrophages), and lymphocytes (e.g. NK and NKT cells) (Gao et al. 2011). These barriers and factors can prevent or destroy the invading pathogens in a non-specific manner; however, recent proofs propose that innate immunity can also specifically detect invading pathogens through pattern-recognition receptors (PRRs) expressed by host cells, which recognize common microbial patterns known as pathogen-associated molecular patterns (PAMPs) (Janeway et al. 2002). Many PAMPs have been identified, including bacterial carbohydrates (e.g., lipopolysaccharide [LPS], mannose) (Seki et al. 2008; Guo et al. 2010), bacterial peptides (flagellin) (Zeng et al. 2006), lipoproteins and nucleic acids (e.g., bacterial or viral DNA or RNA) (Hacker et al. 2002; McCoy et al. 2004; Sander et al. 2011). PAMPs can be recognized by secreted, membrane-bound, or phagocytic PRRs. Complements, pentraxins, and peptidoglycan-recognition proteins are secreted PRRs (Gao et al. 2008; Barreiro et al. 2009). TLRs, nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-induced gene I-like helicases are membrane-bound or intracellular PRRs (Mogensen 2009). Scavenger receptors, macrophage mannose receptors, and β-glucan receptors are known as phagocytic (or endocytic) PRRs (Gao et al. 2011).

The liver plays a key role in the innate immunity response both in host defenses against invading microorganisms and tumor alteration, and in liver injury and repair (Gao et al. 2008; Gao et al. 2011). The biosynthesis of 80–90% of innate immune proteins is due to hepatocytes, including complement components and many secreted PRRs. Hepatic KCs, which represent 80–90% of the total population of tissue macrophages in the body, are responsible, in combination with hepatic sinusoidal cells, for the removal of molecular wastes from the gut.

Furthermore, hepatic lymphocytes are rich in innate immune cells including NK and NKT cells. Moreover, liver non-parenchymal cells also express high levels of membrane-bound PRRs, such as TLRs (Szabo et al. 2006; Seki et al. 2008). The innate immunity in the liver not only plays a key role in host defense against microbial infection and tumor formation but also contributes to sepsis, chronic inflammation, autoimmune diseases, tissue and organ injuries, fibrosis and carcinogenesis (Gao et al. 2011; Seki et al. 2011).

4.2.1 Cell Types in Innate Immune System

Many studies have shown the implication of innate immunity cells including KCs, neutrophils, DCs and NK and NKT in alcoholic liver disease, showing that chronic alcohol intake provokes deregulation in recruitment and functions of inflammatory cells (Gao et al. 2008). Therefore, in the next paragraphs we will discuss the implication of these cell types in ALD.

4.2.1.1 Kupffer Cells

It is well established and accepted that KCs play a key role in the pathogenesis of ALD (Gao et al. 2011; Szabo et al. 2012; Wang et al. 2012). As mentioned above, chronic alcohol intake induces gut barrier permeabilization and consequent LPS translocation to the liver, which provokes KCs activation via TLR4 and triggers inflammatory reactions including the release of pro-inflammatory molecules (Thurman 1998; Wheeler et al. 2001; Cubero et al. 2006).

Activated KCs produce and release pro-inflammatory mediators including TNF-α and ROS, which contribute to hepatocyte necrosis and apoptosis and to the generation of extra-cellular matrix proteins leading to alcoholic liver injury and fibrosis (Cubero et al. 2006; Zakhari 2006). Moreover, the inactivation of KCs using gadolinium chloride has been demonstrated to prevent early alcohol-induced liver injury (Cao et al. 2002; Cubero et al. 2006), underlying the key role of macrophages in ALD.

An important feature of macrophages is their functional plasticity, which is driven by their immunological milieu that contributes to their switch through a wide spectrum of phenotypes, from classical (M1) to an alternative (M2) phenotype (Wan et al. 2013) (Figure 8). M1-polarized macrophages play a key role in several chronic inflammatory diseases including ALD, where clinical findings and experimental data have demonstrated that the impaired release of M1 mediators such as TNF-α, MCP-1, IL-1β and IL-6 contributes to the pathogenesis of several liver lesions including hepatocyte steatosis and apoptosis, inflammatory cell recruitment and fibrogenesis (Louvet et al. 2011; Wan et al. 2013). On the other hand, the inflammation driven by M1 macrophages is counterbalanced by alternatively polarized M2 macrophages, which have been described to promote resolution of inflammation and tissue repair (Louvet et al. 2011; Wan et al. 2013).

Macrophages M2 markers IL-10, Arg1, Ym1 and C-type lectin receptor CD206 (mannose receptor), have been described to play a critical role in suppressing ROS and limiting inflammatory response as well as in promoting tissue repair and remodeling (Titos et al. 2011; Rius et al. 2012).

Moreover, recent evidence showed the presence of protective mechanisms in ALD probably due to M2, which promote M1 apoptosis as protective mechanism against liver injury (Wan et al. 2013).

Altogether, deregulation of the M1/M2 phenotypic balance is emerging as a central mechanism governing the pathogenesis of several chronic inflammatory diseases, suggesting that strategies limiting M1 macrophage polarization and/or favoring the M2 macrophage phenotype may protect against impaired inflammation and consequently limit tissue injury (Murray et al. 2011; Sica et al. 2012; Sica et al. 2013).

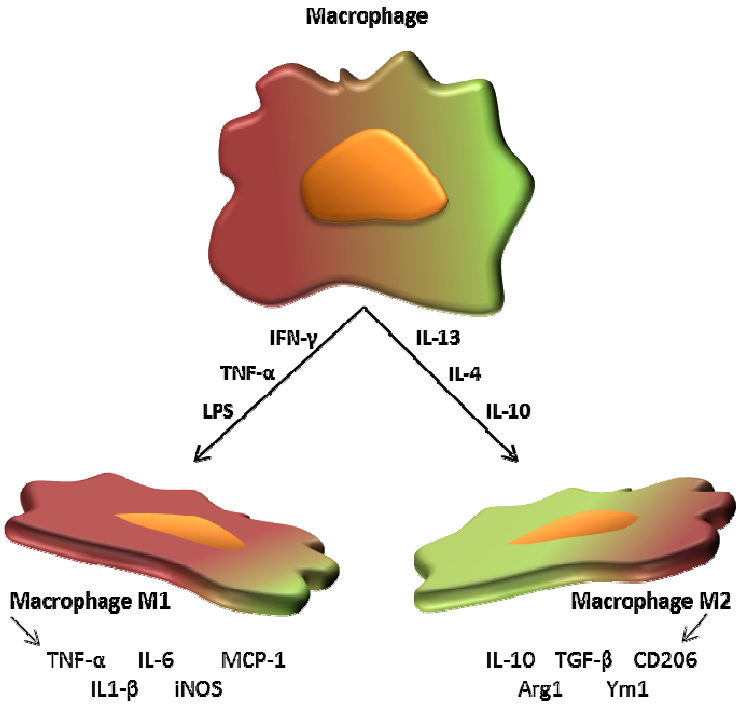


Figure 8. *Scheme of macrophages polarization*

4.2.1.2 Neutrophils

Hepatic neutrophil infiltration is an important response to liver injury, hepatic stress and systemic inflammatory signals. Once neutrophils reach the liver, they can cause mild-to-severe tissue damage and consequent liver failure. Before they reach the liver, neutrophils have to undergo systemic activation (priming), which is mediated by inflammatory molecules such as cytokines, chemokines, complement factors, immune complexes, opsonized particles and other biologically active molecules.

When neutrophils accumulated in the hepatic sinusoids and in post-sinusoidal venules receive signals from damaged cells, they extravasate (transmigrate) into the hepatic parenchyma. Transmigration can be mediated by a chemokine gradient established towards the hepatic parenchyma and endothelial cells, with a major role of intracellular adhesion molecules (ICAM-1). After transmigration, neutrophils adhere to distressed hepatocytes through their β-(2) integrins and ICAM-1 expressed on hepatocytes. Neutrophil contact with hepatocytes mediates oxidative killing of hepatocytes by initiating the respiratory burst and neutrophil degranulation, leading to hepatocellular oncotic necrosis.

Neutrophil-mediated liver injury has been demonstrated in a variety of diseases and chemical/drug toxicities (Jaeschke 2002; Ramaiah et al. 2007). Hepatic neutrophil infiltration is an important hallmark in AH (Jaeschke 2002), however the pathogenic role of these cells remain unclear because the existing experimental models of ALD do not present a prominent neutrophil infiltrate. Recent studies showed some advance in reproducing animal models of AH in mice, demonstrating that chronic-plus-single-binge ethanol feeding synergistically induces liver injury, neutrophil recruitment and fatty liver, which mimics acute-on-chronic alcoholic liver injury as observed in patients (Bertola et al. 2013; Bertola et al. 2013). However, this new data need to be validated and reproduced to show their usefulness in the study of AH and neutrophil infiltration.

Although neutrophil recruitment is one of the most important features of AH in humans, there is no direct evidence that ethanol per se causes its recruitment (Ramaiah et al. 2007). Hepatic steatosis seems to be a pre-requisite to the development of neutrophil inflammation, possibly because a fatty liver is more vulnerable to factors that trigger inflammation (Day et al. 1998). What has been confirmed both in humans and mice is the involvement of bacterial endotoxins, oxidative stress and viral hepatitis in neutrophil recruitment to the damaged liver (Thurman et al. 1998; Ramaiah et al. 2007).

Neutrophil infiltration has been reported as consequence of hepatocytes apoptosis, (Casey et al. 2001; Murohisa et al. 2002) and/or release of inflammatory mediators such as TNF-α, complement factors, vasoconstrictors (such as endothelin-1), adhesion molecules (selectins, LFA-1/Mac-1, ICAM-1, VCAM-1), chemokines and cytokines (Bautista et al. 1992). It is also believed that activated KCs produce and secrete a variety of cytokines and chemokines including IL-8, RANTES, MCP-1, IL-17, that would contribute to recruit neutrophils to the liver (Bautista 2002; Dominguez et al. 2009; Gao et al. 2011). Therefore, targeting these mediators and/or their receptors may provide new potential therapeutic strategies for the treatment of AH.

4.2.1.3 Dendritic Cells

Dendritic cells are the most efficient APCs of the immune system and play a key role in both adaptive and innate immunity. They initiate and regulate immune responses depending on signals received from their specific tissue microenvironments (Rahman et al. 2013).

DCs are sparsely distributed through the liver, and immunohistochemical studies have shown that are mainly present in the portal regions and occasionally in the parenchyma in liver biopsies. Hepatic DCs in both mice and humans, can be divided into two major functional classes: classical DCs (cDCs), expressing high levels of MHCII and functioning as highly-efficient professional antigen presenting cells; and plasmacytoid DCs (pDCs), expressing lower levels of MHCII, which have a limited capacity to capture and present tissue antigens (Rahman et al. 2013). Compared to peripheral DCs, hepatic DCs present a reduced ability to stimulate naive T cells but present increased ability to produce cytokines in response to TLR stimulation (Hsu et al. 2007).

It has been described that hepatic DCs not only act as APCs cells, but also can magnify or ameliorate the hepatocellular damage via producing pro-inflammatory (Connolly et al. 2009) or anti-inflammatory (Bamboat et al. 2010) cytokines. Recent data have shown that hepatic DCs also participate in liver fibrosis regression (Jiao et al. 2011) but not in liver fibrosis progression (Pradere et al. 2013). It is clear that alcohol consumption can modulate DCs function (Gao et al. 2011), but whether DCs directly contribute to the pathogenesis of ALD via production of cytokines remain unknown.

4.2.1.4 NK and NKT Cells

NK and NKT cells, beside with KCs, sinusoidal endothelial cells and HSCs, are the major components of liver sinusoid and play an important role in the pathogenesis of chronic liver diseases and anti-viral and anti-tumoral defense (Seki et al. 2011). Together with KCs and sinusoidal endothelial cells, once activated, resident NK and NKT cells form a strong, innate immune defense system that plays a key role in elimination of pathogens, waste molecules, toxins, and circulating tumor cells from the circulation (Bendelac et al. 2007; Gao et al. 2009).

Hepatic NK cells have been described to be activated in the early stages of virus infection (HCB, HCV) (Ahlenstiel et al. 2009; Amadei et al. 2010), where play a critical role in spontaneous recovery and also contribute to hepatocellular damage by killing hepatocytes (Ahlenstiel et al. 2009; Zhang et al. 2011). In addition to their antiviral function, NK cells also have been described to have anti-fibrotic effects by killing directly activated HSCs that express high levels of NK cell activating ligands (Krizhanovsky et al. 2008; Muhanna et al. 2010; Glassner et al. 2012) and via producing IFN-γ that induce HSCs cycle arrest and apoptosis (Jeong et al. 2006).

Moreover, it has been described that a large number of liver NK cells also express DCs marker such as CD11c, which displayed enhanced cytotoxicity against tumor cells and a greater IFN-γ response compared with $CD11c^-$ NK cells (Burt et al. 2008). By flow cytometry, NKT cells were discovered as NK cells expressing both NK and T cells markers (Bendelac et al. 2007; Gao et al. 2009). Interestingly, the distribution of NK and NKT cells in the livers of mice, rats, and humans has been shown to be different, with a major number of NKT cells in mouse liver compared to rat and human, which on other hand present an increased number of NK cells respect to NKT.

Several evidences in humans and in animal models, showed the inhibitory effects of alcohol consumption on NK cells functions and described multiple mechanisms that contribute to this inhibition such as decreased expression of TRAIL, IFN-γ (Arjona et al. 2004; Jeong et al. 2008), block of bone marrow NK cells release, increased NK spleen cells apoptosis (Zhang et al. 2009) and elevated serum levels of corticosterone (Arjona et al. 2004). Moreover, clinical studies showed that ethanol inhibition of NK cells could represent a key event accelerating hepatitis viral infections, liver fibrosis and liver tumors in patients with AH with hepatic viral infections (Gao et al. 2011).

4.2.2 Endotoxemia and LPS/TLR4 Signaling Pathway

Alcohol consumption is one of the main causes leading to enteric dysbiosis, bacterial over-growth and increased gut permeability. All these factors promote the disruption of the intestinal barrier and result in enhanced gut permeability and consequent translocation of bacteria-derived LPS from the gut to the liver (Gao et al. 2011). These anatomical and functional alterations are responsible for the increased levels of LPS in the blood (endotoxemia), commonly observed in patients with ALD. The liver is the first extra-intestinal organ that encounters venous blood from the small and large intestines via the portal vein. Due to this, the liver is a vulnerable organ to the exposure of bacterial products translocated from the gut lumen via portal vein (Seki et al. 2011).

Gut-derived microbial products play a significant role in the pathogenesis of ALD, and LPS has received special attention since its levels are elevated in plasma of both human alcoholics and in animal models of ALD (Nanji et al. 1993; Fujimoto et al. 2000; Rao 2009). LPS is a component of gram-negative bacteria that biochemically consists of an O-antigen, a core polysaccharide, and a lipid-A component (Lu et al. 2008) and is one of the better characterized PAMPs. The critical role of LPS in alcohol-induced steatohepatitis is believed to be mediated via targeting TLR4 on KCs, the primary hepatic cells that respond to LPS (Szabo et al. 2006; Seki et al. 2008). In response to LPS, KCs produce large amounts of pro-inflammatory cytokines including TNF-α, IL-1, IL-6 and IL-8, which contribute to liver inflammation (Szabo et al. 2006; Seki et al. 2008). Moreover, translocated bacterial products enhance the activation of innate immune cells through PRRs including TLRs, expressed on innate immunity cells (Seki et al. 2008; Wang et al. 2012). The role of TLR4 and its co-receptor CD14 in alcohol-induced liver injury has been well described (Uesugi et al. 2001; Yin et al. 2001; Uesugi et al. 2002) as well as the downstream pathways that contribute to ALD pathogenesis (Hritz et al. 2008; Zhao et al. 2008).

TLR4 specifically recognizes LPS and induces the activation of pro-inflammatory cytokines, chemokines, and transcription factors (Palsson-McDermott et al. 2004; Lu et al. 2008). However, TLR4 is not able to directly bind LPS, to do that needs the help of the adapter molecule MD-2 and its co-receptor CD14 that bring LPS to the receptor complex for recognition by TLR4. Once activated by LPS, TLR4 triggers downstream signaling via its intracellular domain (Palsson-McDermott et al. 2004; Lu et al. 2008; Gao et al. 2011).

Activation of TLR4 induces two downstream pathways: the MyD88-dependent and the MyD88-independent pathway (Figure 9). MyD88-dependent pathway is initiated by recruitment of MyD88

to the TLR4 complex, resulting in downstream activation of Interleukin-1 Receptor-associated Kinase (IRAK)-1/4 and TNF Receptor-associated Factors (TRAF)-6, followed by activation of NF-κB-controlled genes including pro-inflammatory cytokine and chemokines. The MyD88-independent pathway is initiated by recruitment of the TIR-domain-containing Adapter-inducing Interferon-β (TRIF) adapter to the TLR4 complex and results in activation of IKK/TAK1 kinase and IRF-3 phosphorylation as well as late activation of NF-κB (Palsson-McDermott et al. 2004; Lu et al. 2008). Phosphorylated IRF-3 forms a complex that migrates to the nucleus and activates the transcription of IFN-α and β and other interferon-induced genes (Palsson-McDermott et al. 2004; Lu et al. 2008; Gao et al. 2011). It has been shown that the disruption of MyD88 in mice did not prevent alcohol-induced steatohepatitis, ROS production and inflammatory cytokines in the liver (Hritz et al. 2008); whereas the disruption of TRIF (molecule belonging to the MyD88-indipendent pathway) abolished alcohol-induced steatohepatitis (Zhao et al. 2008), suggesting that MyD88-indipendent signaling plays a key role in TLR4-mediated alcoholic liver injury.

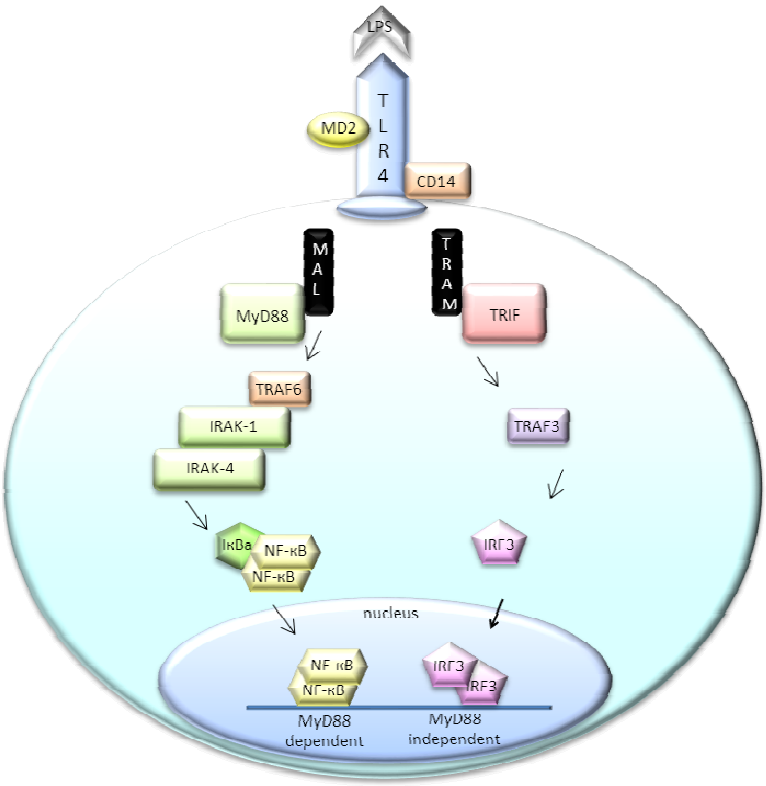


Figure 9. *LPS/TLR4 Signaling: MyD88-dependent and MyD88-independent pathways*

4.2.2.1 LPS/TLR4 in Alcoholic Liver Disease

LPS-TLR4 signaling in HSCs, has been shown to play an important role in the pathogenesis of liver fibrosis (Seki et al. 2007) by enhancing TGF-β signaling and by producing cytokines and chemokines that contribute to liver fibrogenesis (Figure 10) (Paik et al. 2003; Seki et al. 2007).

Quiescent HSCs are resistant to TGF-β-induced activation due to the high expression levels of Bambi, which inhibits TGF-β receptor signaling (Seki et al. 2007). Upon TLR4 activation, Bambi expression is quickly down-regulated, leading to TGF-β receptor signaling activation. LPS-TLR4 signaling pathway activation in HSCs, also induce the production of CC chemokines, including MCP-1 and RANTES (Bataller et al. 2005; Seki et al. 2007). These chemokines contribute to HSCs activation and consequently to hepatic inflammation and fibrogenesis promotion by binding and activating their CC receptors.

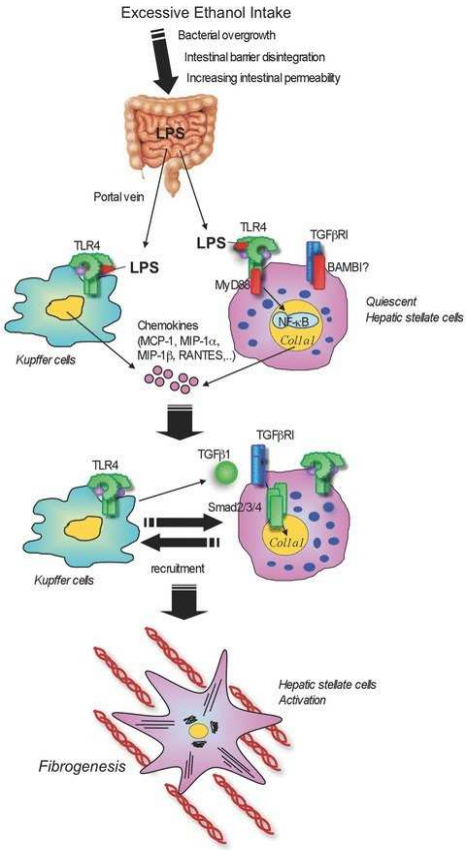


Figure 10. *Bacterial translocation and hepatic TLR4 signaling in alcoholic liver fibrosis (From Seki, E and B. Schnabl 2011)*

4.2.3 Complement System Activation

Recent evidences suggest the implication of complement system activation in the pathogenesis of numerous liver diseases, including ALD via the production of inflammatory cytokines (Qin et al. 2006; Gao et al. 2011). The complement system is an ancient part of the immune system that links innate and adaptive immunity (Gao et al. 2011) and comprises more than 30 proteins, the majority of them produced and secreted by the liver. Complement can be activated via three pathways: classical, lectin and alternative pathway (Figure 11). These three pathways converge to the third part of the complement (C3), which is cleaved by convertases and results in C3a and C3b (Gasque 2004). Hepatic cells express complement factor receptors and intrinsic regulatory proteins, specifically, it has been described that KCs and HSCs under basal conditions express the anaphylatoxin C3a and C5a receptors (Qin et al. 2006) and that hepatocytes also express C5s receptor when are proliferating or in response to inflammatory cytokines (Qin et al. 2006; Gao et al. 2011).

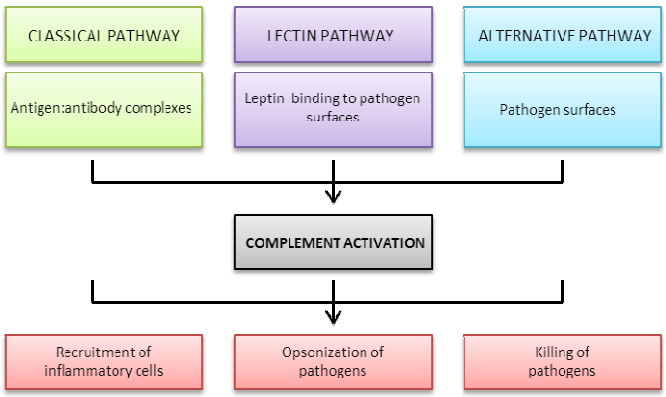


Figure 11. *Schematic overview of the complement cascade(Adapted from Janeway et al. 2001)*

It has also been described that alcohol intake leads to an early activation of the complement, resulting in C3 and C5 cleavage. Activated C3a and C5a interact with their receptors on KCs and HSCs leading to TNF-α production and consequent hepatocyte damage (Qin et al. 2006; Gao et al. 2011) (Figure 12).

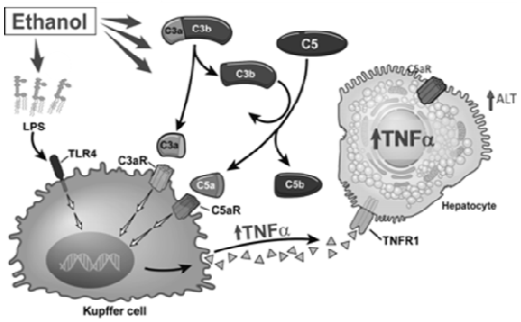


Figure 12. *Activation of the complement system contributes the pathogenesis of ALD (From Gao et al. 2011)*

5. CYTOKINES AND CYTOKINE-RECEPTORS IN ALCOHOLIC LIVER DISEASE

Cytokines are pleiotropic, regulatory molecules that elicit their effect by binding and activating specific cytokine-receptors. Cytokines can be virtually produced by every nucleated cell of the body, including all types of liver cells. In most tissues, including the liver, constitutive production of cytokines is minimal or absent (Tilg 2001; Tilg et al. 2006). The production of these effector molecules is a consequence of cells activation in physiologic and pathologic conditions (Tracey et al. 1993; Tilg et al. 2006).

There is increasing evidence supporting a major role for several cytokines in various aspects of inflammatory liver diseases and liver tissue repair (Table 1). Cytokines have been proposed as important mediators of hepatic inflammation, liver-cell death, cholestasis (Trauner, Meier et al. 1998; Friedman 2000; Tilg and Diehl 2000) and also regeneration of the damaged liver (Cressman, Greenbaum et al. 1996; Tilg, Kaser et al. 2006); in most cases having a profound impact on the outcome of the disease (Tilg 2001). The cytokine family consists of several subfamilies including: the interleukins (ILs), the TNF family, IL-6 and IL-6-related cytokines, interferons (IFNs), chemokines, transforming growth factor-β (TGF-β) among others (Tilg, Kaser et al. 2006).

Table 1. Key properties of cytokines involved in liver disease (Adapted from Tilg et al. 2006)

Pro-inflammatory Cytokines	Properties
IL-1α, IL-1β, TNF-α	Stimulation of acute phase protein synthesis
IL-6, IL-11, oncostatin M, cardiotrophin	Pro- and anti- inflammatory activities, Stimulate acute phase proteins, Regulate hepatic regeneration and Ig synthesis
IFN-γ	Immunoregulatory Th1 cytokine, Induce TNF-α, Recruit mononcytes and neutrophils to site of inflammation
IL-12	Th1-1-directing cytokine
IL-18	IFN-γ-inducing factor, Pro-inflammatory in early stages of immune response

Anti-inflammatory Cytokines	Properties
IL-1 receptor antagonist	Blocks binding of IL-1 to cell-surface receptors
Soluble IL-1 receptor type II	Binds circulating IL-1
Soluble tumor necrosis factor receptor (TNFR) p55(I)/p75(III)	Block TNF-α-regulated inflammatory processes, comprise domains of TNFRp55 and p75
IL-18 binding protein	Neutralizes IL-18
IL-10	Regulates B-cell function, Inhibits TNF-α
IL-4, IL-13	Th-2 cytokines,

	Regulate B-cell function, Suppress synthesis of pro-inflammatory cytokines
IL-6	Hepatoprotective- protects against ALD via STAT3
IL-22	Hepatoprotective- protects against acute and chronic ALD by binding to a receptor complex composed of IL-10R2 and IL-22R chains on hepatocytes
Adiponectin	Adipokine, Induces anti-inflammatory cytokines, Suppresses endotoxin induced TNF-α expression

Cytokines in Immune Responses	Properties
IL-2, IL-4, IL-7, IL-9, IL-15	Interleukins, Tcells differentiation and maturation
IL-2, IFN-γ	Th-1 cytokines, Direct anti-viral response, Pro-inflammatory
IL-4, IL-5, IL-10	Th-2 cytokines, Mediate inflammation, Allergic responses, Immunoglobulin synthesis

Cytokines in Acute Liver Failure	Properties
TNF and TNFR p55/p75	Liver failure
Fas, Fas Ligand	Death receptors, Involved in liver injury and apoptosis
IL-18	Mediates TNF- and Fas-related experimental liver failure

Anti-fibrogenic Cytokines	Properties
Hepatocyte growth factor (HGF)	Anti-fibrogenic, Anti-apoptotic, Promotes liver regeneration
Fibrogenic Cytokines	**Properties**
TGF-β	Induced by pro-inflammatory cytokine Promotes fibrogenesis
PDGF	Promotes proliferation and migration of HSCs

5.1 Cytokines in Alcoholic Liver Disease

Experimental and human studies support the role of cytokines in the pathogenesis of ALD. It has been demonstrated that patients with ALD show high levels of IL-1, -6, and -8, as well as TNF-α and MCP-1. Moreover, elevated levels of several of these cytokines in blood have been correlated with poor prognosis and increased mortality in patients with AH.

It is well established that ALD is associated with imbalanced immune responses and increased production of pro-inflammatory cytokines (Tilg et al. 2000; McClain et al. 2004). Moreover, cytokines display a wide range of effects not only in the modulation of inflammatory responses, but also actively participating in liver fibrosis progression and perpetuation. In ALD, the increased production of cytokines is mostly due to LPS-activated Kupffer cells and ethanol-induced activation of the classical complement pathway, both leading to an early increase in the expression of pro-inflammatory cytokines that contribute to the pathogenesis of the disease. It has been shown that once activated, KCs produce ROS and TNF-α via MyD88-independent and TRIF-dependent pathways (Hritz et al. 2008; Zhao et al. 2008) but also produce hepatoprotective (such as IL-6) and anti-inflammatory (such as IL-10 and adiponectin) cytokines that play compensatory roles in ameliorating ALD (Miller et al. 2011).

5.1.1 The Tumor Necrosis Factor Superfamily

The TNF superfamily includes a large number of ligands and receptors, which are involved in the response to liver damage. This cytokines cytokine-receptors group has attracted substantial interest as a potential source of therapeutic targets for the management of human diseases. TNF superfamily ligands are primarily expressed as type II transmembrane proteins, and in some cases are processed into smaller, secreted proteins that retain biological activity (Locksley et al. 2001; Bodmer et al. 2002). Both anchored and soluble, cytokines contain a C-terminal TNF homology domain that mediates self-trimerization and receptor binding. TNF superfamily members bind to one or more members of the TNF receptor (TNFR) superfamily, most of which are type I, or type III transmembrane proteins (Winkles 2008). TNFR are characterized by the presence of an extracellular, ligand-binding region containing one to four cysteine-rich domains and a cytoplasmic tail containing one or more adaptor-protein binding sites.

The TNF superfamily contains a variety of cytokines and receptors which activate signaling pathways of cell survival, death and differentiation. The most relevant ligands of this superfamily include TNFα, Fas ligand and TNF-like weak inducer of apoptosis (TWEAK) which have been implicated in liver damage and remodeling.

TNF-α is a pivotal cytokine involved in inflammation and, in the liver, is produced primarily by KCs. It has been shown that inactivation of KCs prevents alcohol-induced liver injury; and that the hepatic inflammation and necrosis observed in ethanol-fed rats were attenuated significantly by antibody treatment. These results support the hypothesis that TNF-α plays an important role in inflammation and necrosis in alcohol-induced liver injury and that treatment with anti-TNF-α antibody may be therapeutically useful in this disease (Iimuro et al. 1997). Few years ago, based on these promising evidences, it was proposed that a potential therapy for ALD may be agents that down-regulate TNF production or block TNF activity. Indeed, agents such as prostaglandins and glucocorticoids (both inhibiting TNF production) have been used in both human liver disease and experimental models of liver injury, and anti-TNF antibody has been shown to attenuate the hepatotoxicity in an animal model of alcoholic-related liver disease (McClain et al. 1998). However, drugs interfering TNFα were tested in these patients but the results were disappointing due to an increased incidence of severe bacterial infections and mortality. Therefore, an important purpose in cytokine research is to develop effective strategies to control over-production of cytokines while preserving their beneficial effects.

5.1.2 TWEAK/Fn14 Interaction

TWEAK, also known as TNFSF12, APO3L, CD255 , is a ligand belonging to the TNF superfamily described to elicits its effects by binding and activating Fn14 receptor.

TWEAK, in contrast to other TNF superfamily members (including TNF-α), is expressed in many different tissues and tumor specimens (Tran et al. 2003; Ho et al. 2004; Kawakita et al. 2005; Winkles 2008). Moreover, TWEAK has been shown to regulate cell proliferation, migration, survival, cell death and differentiation when added to either human, mouse or rat primary cells or to immortalized cell lines cultured *in vitro* (Table 2) (Winkles 2008). In most of these studies it has been reported that recombinant human soluble TWEAK, can bind to the mouse and rat Fn14 receptors. TWEAK–Fn14 human and mouse cross-reactivity has been also confirmed biochemically (Bossen et al. 2006), and TWEAK and Fn14 amino-acid sequences have been shown to be remarkably conserved throughout evolution (Chicheportiche et al. 1997; Brown et al. 2006; Glenney et al. 2007)

Table 2. TWEAK effects on different cell lines (From Winkles 2008)

Cellular response (stimulate (+) or inhibit (–))	Cell type(s)
Proliferation (+)	Human endothelial cells
	Human smooth muscle cells
	Human liver tumour cell lines
	Murine EpH4 mammary epithelial cells
	Murine C2C12 myoblasts
	Murine primary myoblasts
	Murine primary astrocytes
	Murine synovial cells
	Rat liver NRC-1 cells
Proliferation (–)	Murine postnatal neural progenitor cells
Migration (+)	Human endothelial cells
	Human glioma cell lines
	Rat aortic smooth muscle cells
Survival (+)	Human endothelial cells
	Human glioma cell lines
Differentiation (+)	Murine RAW264.7 monocytes → osteoclasts
Differentiation (–)	Human immature erythroblasts → mature erythroblasts
	Human mesenchymal stem cells → chondrocytes
	Human osteoblast precursors → osteoblasts
	Murine MC3T3-E1 cells → osteoblasts
	Murine C2C12 myoblasts → myotubes*
Death (+)	Human HT-29 tumour cell line‡
	Human KATO-III tumour cell line‖
	Human MCF7 tumour cell line§
	Human HeLa tumour cell line§
	Human Kym-1 tumour cell line‖
	Human HSC3 tumour cell line
	Human SW480 tumour cell line
	Human peripheral blood mononuclear cells
	Murine mesangial cells‖
	Murine renal MCT cells¶
	Murine primary cortical neurons

The Fn14 receptor (also known as TNFRSF12A, TWEAKR, CD266), is the smallest TNFR superfamily member described. TWEAK is the only TNF superfamily member that has been shown to bind Fn14 (Bossen et al. 2006). The Fn14 cytoplasmic tail, contains a single TRAF consensus binding motif and TRAF1, TRAF2, TRAF3 and TRAF5 are able to bind this site (Brown et al. 2003; Han et al. 2003).Fn14 has been detected in many cell and tissue types, as

well as its ligand TWEAK. The only Fn14-negative cells described until now, are primary T and B cells (Nakayama et al. 2002; Maecker et al. 2005) and their corresponding immortalized cell lines. One interesting aspect of Fn14 biology, which is not shared by any other member of the TNFR superfamily, is that *Fn14* gene expression is highly regulated both *in vitro* and *in vivo* (Meighan-Mantha et al. 1999; Feng et al. 2000) and several growth factors, cytokines and hormones enhance Fn14 expression (Table 3).

Table3. *Substances that induce Fn14 expression in vitro (From Winkles 2008)*

Agent	Cell type
Angiotensin II	Rat aortic smooth muscle cells
Bone morphogenetic protein 6	Murine intra-embryonic endothelial cells
Epidermal growth factor	Murine NIH 3T3 fibroblasts
	Rat aortic smooth muscle cells
Fetal bovine serum	Human M426 fibroblasts
	Human aortic smooth muscle cells*
	Murine NIH 3T3 fibroblasts
	Rat aortic smooth muscle cells
Fibroblast growth factor 1	Human M426 fibroblasts
	Murine NIH 3T3 fibroblasts
Fibroblast growth factor 2	Human umbilical vein endothelial cells
	Murine NIH 3T3 fibroblasts
	Rat aortic smooth muscle cells
Interferon-γ	Human aortic smooth muscle cells*
	Human immature erythroblasts
	Human $CD14^+$ monocytes
	Human natural killer cells
	Human dendritic cells
	Murine renal MCT cells
Interleukin-1β	Human aortic smooth muscle cells*
	Human gingival fibroblasts
Bacterial lipopolysaccharide	Human THP-1 monocytic cells
Nerve growth factor	Rat PC12 cells
Platelet-derived growth factor-BB	Murine NIH 3T3 fibroblasts
	Rat aortic smooth muscle cells
Phorbol 12-myrisate 13-acetate	Human M426 fibroblasts
	Human $CD14^+$ monocytes
	Human natural killer cells
	Human dendritic cells
	Murine NIH 3T3 fibroblasts
	Rat aortic smooth muscle cells
Transforming growth factor-β1	Human gingival fibroblasts
	Murine NIH 3T3 fibroblasts
Thrombin	Rat aortic smooth muscle cells
Tumour necrosis factor-α	Murine renal MCT cells
TNF-like weak inducer of apoptosis	Human glioma cells
Vascular endothelial growth factor A	Human umbilical vein endothelial cells

Observation that serum, which *in vivo* is present only at sites of tissue injury and remodeling, was a potent inducer of *Fn14* expression *in vitro (Meighan-Mantha et al. 1999; Feng et al. 2000; Winkles 2008)*, suggested that *Fn14* expression might be increased after tissue injury. Data from murine partial hepatectomy (Feng et al. 2000) and rat artery balloon injury models, confirmed this hypothesis (Wiley et al. 2001) and further studies showed that Fn14 gene activation also occurs in response to other tissue insults (Figure 13). These findings, together with two independent studies demonstrating Fn14 induction during nerve regeneration (Tanabe et al. 2003; Fischer et al. 2004) and the results obtained using genetically engineered mice, confirmed that the TWEAK–Fn14 axis contributes to adult tissue repair and remodeling.

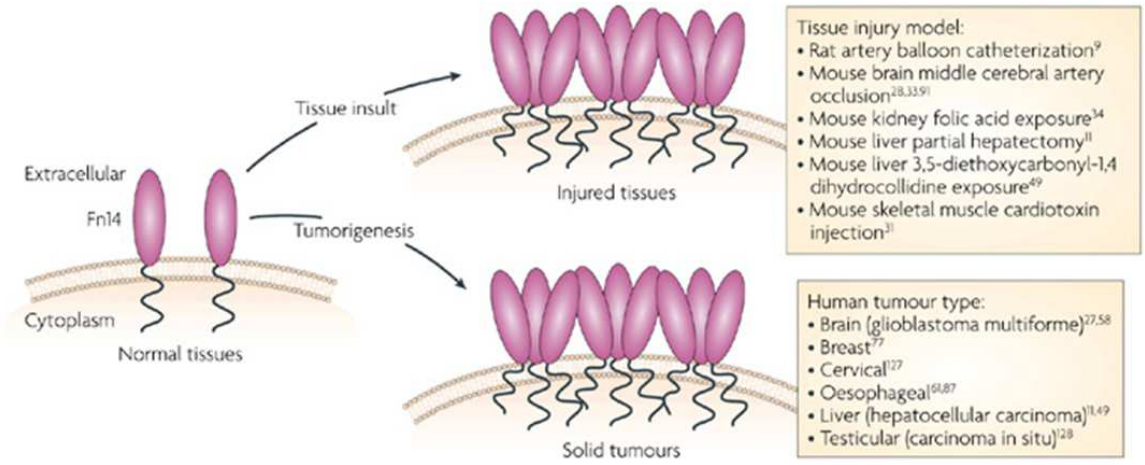


Figure 13. *The Fn14 gene expression in vivo (From Winkles 2008)*

5.1.3 TWEAK-Dependent and -Independent Fn14 Signaling

As for every TNF ligand and respective TLR receptor, when TWEAK binds to its receptor Fn14, it induces receptor trimerization, TRAF association with the cytoplasmic tail and activation of intracellular signaling cascades (Locksley et al. 2001; Bodmer et al. 2002) (Figure 14). Indeed, it is known that TWEAK activation of intracellular signaling pathways requires the Fn14 TRAF-binding motif (Saitoh et al. 2003) and TRAF2 and TRAF5 function (Saitoh et al. 2003; Winkles 2008).

TWEAK treatment of Fn14-positive cells has been shown to activate several different signaling cascades. NF-κB pathway activation seems to be a common cellular response, for which it has been reported that TWEAK-Fn14 binding activates the canonical NF-κB signaling pathway. Moreover, the treatment of various cell types *in vitro* with TWEAK has been shown to induce the expression of

known NF-κB target genes including the secreted MMP9, the anti-apoptotic proteins A20, BCL-2 and BCL-XL, TRAF1 and TRAF3 and Fn14 itself (Tran et al. 2005; Girgenrath et al. 2006; Tran et al. 2006). Most of the other TWEAK-responsive genes identified to date encode inflammation-associated proteins including cytokines such as IL-6; chemokines such IL-8, MCP-1 and RANTES; and cell-cell adhesion molecules such ICAM-1 and VCAM-1 (Harada et al. 2002; Campbell et al. 2006; Girgenrath et al. 2006).

Fn14 has been reported to be able to signal in a ligand-independent manner too, when it is ectopically overexpressed *in vitro*. Specifically, ectopic Fn14 expression has been shown to activate the NF-κB signaling pathway (Brown et al. 2006) and induce cellular responses. These Fn14-triggered effects require an intact TRAF binding site in the Fn14 cytoplasmic tail (Brown et al. 2006). Thus, it is possible that TWEAK-independent Fn14 signaling can occur when intracellular Fn14 levels reach a certain threshold level. The explanation of this event could be that Fn14 up-regulation induces spontaneous trimerization and multimerization, and that this 'receptor clustering' promotes TRAF association and intracellular signaling cascade activation (Winkles 2008) (Figure 14).

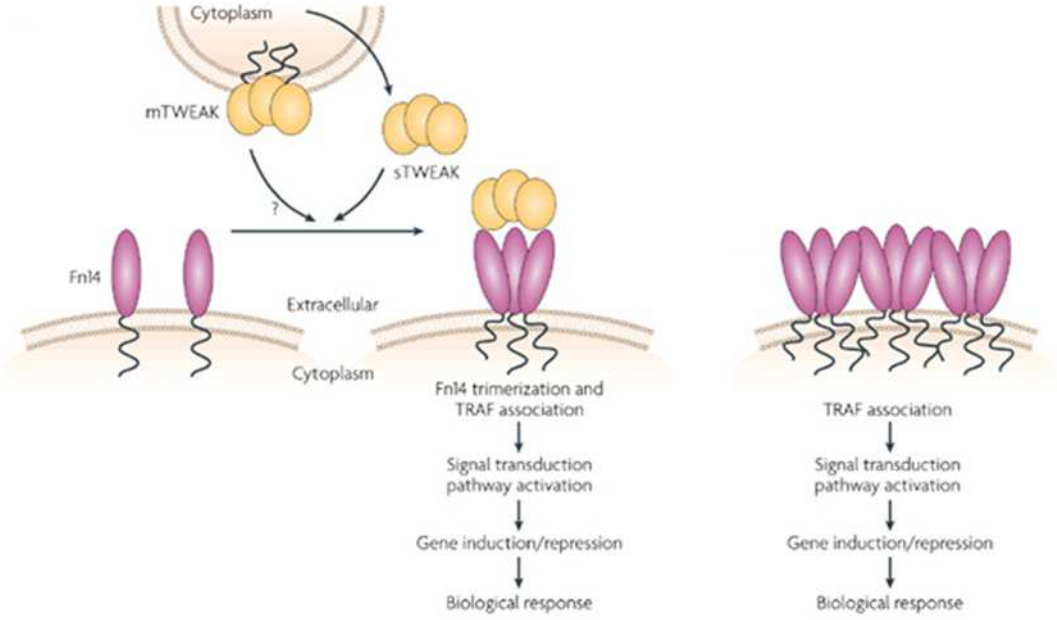


Figure 14. *TWEAK-dependent and –independent Fn14 signaling (From Winkles 2008)*

As mentioned before, Fn14 is expressed at high levels in injured tissues and in solid tumors, but these levels of expression may or may not be high enough to trigger ligand-independent signaling. If these Fn14 expression levels are sufficient, then the TWEAK expression level could be the critical factor that controls whether ligand-dependent or -independent Fn14 signaling predominates (Figure 15). In case of low TWEAK expression and high Fn14 expression, as how has

been reported in advanced brain tumors (Tran et al. 2003), then TWEAK-independent Fn14 signaling may predominate.

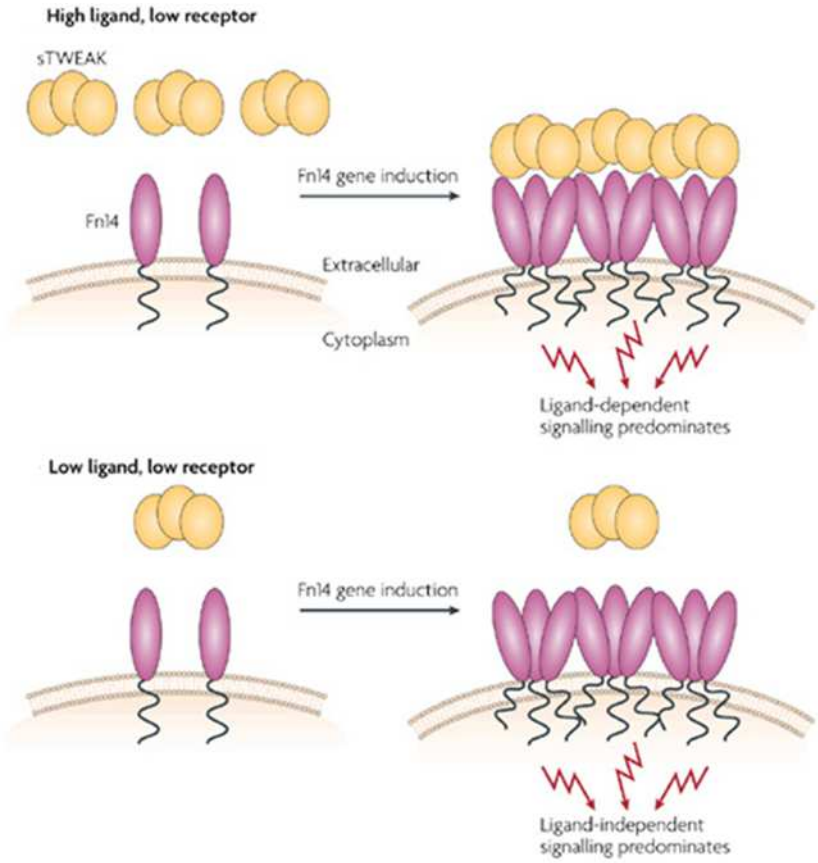


Figure 15. *TWEAK- Fn14 cytokine-receptor axis (From Winkles 2008)*

5.1.4 TWEAK and Fn14 as Potential Therapeutic Targets

It has been described that TWEAK-dependent and -independent Fn14 signaling, might have an important role in wound repair, a complex process involving blood clotting, cell proliferation, cell migration, inflammation and angiogenesis (Werner et al. 2003). Despite in acute injury, Fn14 signaling would be transient and beneficial, in conditions of chronic tissue injury and inflammation, TWEAK and/or Fn14 levels may be elevated, which could result in persistent activation of Fn14-coupled intracellular signaling cascades and this would probably produce dangerous and pathological effects (Winkles 2008). Moreover, Fn14 has also been described to be expressed by progenitor cells in damaged tissues (Jakubowski et al. 2005) thus, therapeutic targeting strategies aiming to inhibit TWEAK-dependent and -independent Fn14 signaling could represent an important approach in the clinical setting for the treatment of acute and chronic diseases.

5.2 Chemokines in Liver Inflammation and Fibrosis

Chemokines are small heparin-binding chemotactic cytokines (7 to 13 KD), which have been described to orchestrate immune responses during acute and chronic tissue damage. Besides modulating immune cell trafficking, chemokines also regulate important biological processes including hematopoiesis, cardiogenesis, vasculogenesis and neuronal development (Sahin et al. 2012).

The chemokine network, consisting of at least 50 ligands and 19 receptors, is a highly redundant and promiscuous system (Sahin et al. 2012). This promiscuity has long been seen as a limitation for investigating the specific effects of single chemokines, as ligands of the same receptor have *in vitro* and *in vivo* specific effects. However, knocking-down technologies have recently demonstrated that the chemokine complex is not as redundant as originally thought and that the deletion of single chemokines or receptors produces distinct effects (Rot et al. 2004; Bonecchi et al. 2009) allowing their study.

The members of chemokine family share similar structures and can be divided in four groups: CC, CXC, CX3C and C characterized by the number of amino acids located between the N-terminal cysteine residues (Figure 16) (Rot et al. 2004; Bonecchi et al. 2009). Chemokines bind specific seven transmembrane G-protein coupled receptors, selectively expressed on different immune and non-immune cell types. The receptors are also divided into four families: CCR, CXCR, CX3CR and XCR, in accordance to chemokines classification. Although several ligands may bind to a single receptor, ligands of a specific family can bind only receptors belonging to the same family.

Within the variety of molecules playing a critical role in tissue inflammation and fibrosis, chemokine receptors are attractive drug targets from a pharmacological point of view (Pease et al. 2009; Pease et al. 2009). A CCR5 antagonist (Maraviroc, Pfizer) has already been licensed for use in humans for a second-line treatment of HIV infection (Sayana et al. 2009). Although CCR agonists have not yet been applied to liver diseases, the evolving knowledge of chemokine effects within the liver provide strong basis for investigating in this direction.

In recent years chemokines have emerged as key mediators of the inflammatory response in many acute and chronic liver diseases. In addition to their role in inflammatory cell recruitment, chemokines have also been shown to directly influence the function of liver resident cells such as HSCs, KCs and hepatocytes, participating in different inflammatory and fibrogenic pathways within

the liver. Further investigation of chemokines will provide new insights in the molecular pathogenesis of liver inflammation and fibrosis.

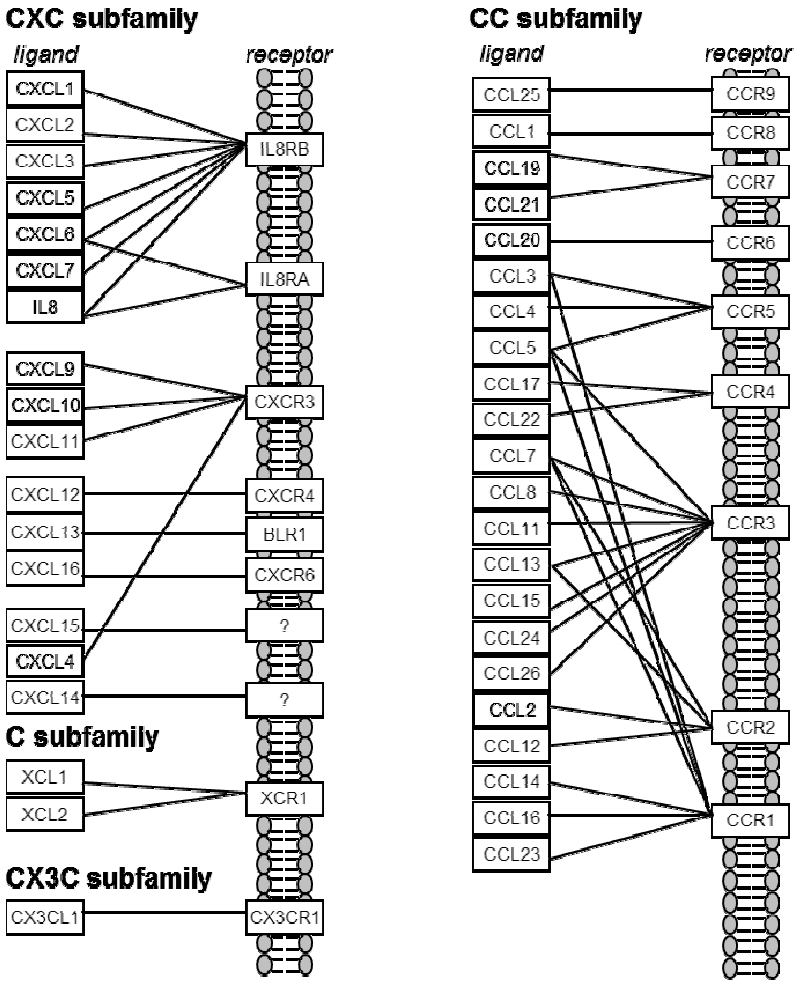


Figure 16. *Chemokines and chemokines receptors (Adapted from Affò et al. 2013)*

5.2.1 CXC Chemokines in Liver Inflammation and Fibrosis

The CXC chemokines are characterized by a non-conserved single amino acid between the N-terminal cysteine residues (Wasmuth et al. 2010). This subfamily presents a typical glutamate-leucine-arginine (ELR^+) motif near the protein N terminus (Baggiolini 2001), which has been proven to be crucial to attract neutrophils to the sites of injury and inflammation and in the induction of other cytokines such as TNF-α and IL-1 (Wasmuth et al. 2010). Therefore, these chemokines likely play an important role in the early phases of inflammation and wound repair in the injured liver.

In liver samples from patients with AH, it has been demonstrated that the expression of the CXC family members IL-8, Gro-alpha, CXCL5, CXCL6, CXCL10, and CXCL4 is up-regulated compared

to controls and that the higher expression levels of IL-8, CXCL5, Gro-gamma, and CXCL6 is associated with worse prognosis. Moreover, IL-8 protein levels have been shown to be an independent predictor of 90-day mortality in patients with AH (Dominguez et al. 2009).

It has been recently shown that CXCR3 is involved in the positioning of Th1 and Treg cells into the liver during liver fibrosis (Santodomingo-Garzon et al. 2009). This receptor binds the IFN-γ inducible chemokines CXCL9, CXCL10, CXCL11 and CXCL4 in humans (Lasagni et al. 2003). Mice lacking CXCR3 are more prone to develop liver fibrosis, which is initiated by the loss of anti-fibrogenic and angiostatic effects of CXCL9 on HSCs (Wasmuth et al. 2009) and sinusoidal endothelial cells (Sahin et al. 2012), supporting the hypothesis that angiogenesis also promote the fibrotic process (Fernandez et al. 2009).

In contrast, the deletion of CXCL10 (another ligand of CXCR3), has been shown to inhibit experimental liver fibrosis (Hintermann et al. 2010). These results are in accordance with clinical studies showing high serum levels and intra-hepatic expression of CXCL10 in severe HCV induces fibrosis.

In line with the results obtained with CXCL10, deletion of CXCL4, another ligand of CXCR3, protected mice from experimental liver fibrosis development, due to an impaired recruitment of $CD8^+$ T cells and reduced CXCL4-induced pro-inflammatory chemokines (Zaldivar et al. 2010). These results suggest that the development of molecules targeting CXC chemokines might be an interesting therapeutic approach to modulate hepatic inflammation and fibrosis.

5.2.2 CC Chemokines in Liver Inflammation and Fibrosis

CC chemokines are the largest family of all known chemokines (Bonecchi et al. 2009) and most of its members have been detected at sites of active inflammation and tissue injury. Many studies have shown that CC chemokines are major attractants for T cells, B cells, monocytes, eosinophils and basophils (Charo et al. 2006).

One of the most investigated and best-characterized CC chemokine within the liver is CCL2 (MCP-1). CCL2 is secreted by hepatocytes, KCs, biliary epithelial cells after toxic or biliary damage and by HSCs (Marra et al. 1993; Marra et al. 1995; Kruglov et al. 2006). Indeed, CCL2 was the first chemokine shown to be produced by activated as well as quiescent human HSCs after stimulation with different cytokines (Holt et al. 2009), thereby supporting the contribution of HSCs to immune

responses within the liver. CCL2 binds to CCR2 receptor, which is present on a variety of cells including monocytes, immature DCs, Th1 cells and HSCs (Seki et al. 2009). In accordance with the high expression of CCL2 in the liver after toxic or biliary injury, CCR2 deficient mice have been shown to be protected from liver fibrosis in the carbon tetrachloride (CCl_4) and bile duct ligation experimental models of liver fibrosis (Seki et al. 2009).

CCL5/CCR5 is another important and well-described chemokine chemokine-receptor axis that has been largely investigated in the liver. Gene expression analysis showed high levels of CCL5 in patients with chronic liver diseases (Nischalke et al. 2004; Seki et al. 2009) and in animal models of liver fibrosis. Moreover, mice with genetic depletion of CCR5 and CCR1 (another receptor of CCL5), have been shown to be significantly protected from liver fibrosis during chronic liver injury. These results showed that CCR1 induce its pro-fibrogenic effects through hematopoietic cells and CCR5 contribute to liver fibrosis acting on resident liver cells (Nischalke et al. 2004; Seki et al. 2009). *In vitro* experiments in HSCs of human and murine origin also demonstrated that CCL5-induce HSCs migration depends on ROS formation and Akt and ERK signaling (Schwabe et al. 2003). Interestingly, CCL5 has been also shown to be able to induce HSCs proliferation, but only in human HSCs, not in murine cells (Seki et al. 2009), highlighting species differences in reactivity to chemokines, as well as remarking that HSCs show different activation and gene signatures when isolated from healthy or injured liver (De Minicis et al. 2007).

5.2.2.1 CCL20 Chemokine

Human CCL20 was simultaneously reported by three different groups as the first chemokine discovered through bioinformatics-based searches of DNA databases and automated sequencing techniques (Schutyser et al. 2003). Screening the public GenBank EST database with amino acid and nucleotide sequences of various known CC chemokines, Hieshima et al. identified five partial, overlapping cDNA sequences (three from pancreatic islets, one from fetal lung and one from HepG2 cells from hepatocarcinoma) encoding for a novel human CC chemokine (Hieshima et al. 1997). The full-length cDNA was cloned from monocytic U937 cells and the sequence (799 basepairs) encoded a protein of 96 amino acid residues and its existence was further confirmed by the isolation of three independent cDNA clones from a human liver cDNA library (Hieshima et al. 1997). Corresponding mRNA was mainly expressed in the liver and induced in some human cell lines. Therefore, this CC chemokine was first designated liver and activation-regulated chemokine (LARC).

Meanwhile, Rossi et al. retrieved four of these CCL20-encoding ESTs (Rossi et al. 1997). They cloned the full-length cDNA from a human activated monocyte cDNA library and called this novel CC chemokine macrophage inflammatory protein-3 α (MIP-3α) based on its structural relationship with known members of the CC chemokine family. Simultaneously, Hromas et al. cloned an EST with some homology to known CC chemokines from a cDNA library prepared from human pancreatic islet cells (Hromas et al. 1997). Based on its common property with CC chemokine family members to chemoattract peripheral blood mononuclear cells, this CC chemokine was termed Exodus-1. In the systematic chemokine nomenclature, LARC/MIP-3α/Exodus-1 is designated as CCL20 (CC chemokine ligand 20) (Zlotnik et al. 2000). A murine, rat and rhesus macaque homologue for human CCL20 has been also identified (Varona et al. 1998), (Utans-Schneitz et al. 1998) (Basu et al. 2002).

In contrast to the first-generation CC chemokines genes, all grouped at chromosome 17q11.2, the human *CCL20* gene was mapped to chromosome 2q33-37 (Schutyser et al. 2003). Although intron/exon junctions of the CC chemokine subfamily members are normally highly conserved, with the majority containing three exons and two introns, the CCL20 gene consist of four exons and three introns and is an exception to this general rule (Nelson et al. 2001).

Constitutive expression of CCL20 has been demonstrated in several normal human tissues including fetal liver and lung and in many cell types including lung macrophage and dendritic cells. Moreover, it has been described that CCL20 can also be induced in different cell types including fibroblasts and monocytes, when incubated with pro-inflammatory substances such as IL-1β, TNF-α and LPS (Schutyser et al. 2003).

CCL20 has been described to elicit its effects by binding and activating its selective transmembrane G-protein coupled receptor CCR6 (Schutyser et al. 2003). CCR6 was demonstrated to be constitutively expressed in both lymphoid and non-lymphoid organs: predominantly in spleen, lymph nodes, appendix and pancreas, and to a lesser degree in peripheral blood leukocytes, thymus, small intestine, fetal liver and testis. CCR6 is mostly expressed by immature dendritic cells and memory T cells and has also been described to be important for B-lineage maturation.

The fact that CCL20 is expressed in a broad spectrum of cells and tissue types in normal conditions and that CCL20 may be up-regulated as a result of the effects of a variety of CCL20-inducing agents, suggest that CCL20 and CCR6 are involved in both normal and pathological processes. A potential role of CCL20 and CCR6 has been suggested in skin, mucosal immunity,

cancer -including hepatocellular carcinoma (Chen et al. 2011) and in rheumatoid arthritis (Schutyser et al. 2003).

Only recent studies focused their attention on CCL20 and CCR6 in hepatic liver diseases. High levels of CCR6 have been detected in Th17 intrahepatic cells and CCL20 has been reported to be expressed by biliary epithelial cells and increased in this cell type in response to cytokine treatment (Oo et al. 2012). Recently, Hammerich et al. have described the potential role of CCR6 in chronic liver disease (Hammerich et al. 2013). Using liver samples of patients with different stages of chronic hepatic injury, hepatic expression of both CCL20 and CCR6 was found significantly up-regulated in these patients. Nevertheless, further studies are needed to demonstrate the role of chemokine CCL20 in the pathogenesis of liver diseases and the mechanisms leading to its up-regulation.

AIMS

The global strategy of this thesis is based in the hypothesis that performing transcriptome analysis of livers from patients with AH, we will be able to identify genetic drivers and pathways involved in the pathogenesis of AH, which may provide important insights for the development of new therapeutic strategies.

Therefore, the overall aim of this thesis was to identify novel molecular drivers of AH and to investigate their role in the pathogenesis of AH in order to reveal new potential targets for therapy.

Specifically the main aims of this thesis were:

1. **To identify new targets for therapy in AH through a transcriptome analysis of human liver samples from patients with AH.**

   1.1 To perform a transcriptome analysis in liver samples of patients with AH.
   1.2 To identify genes and pathways differentially regulated in patients with AH and potentially implicated in the pathogenesis of the disease.
   1.3 To analyze the cytokine-cytokine receptor interaction pathway in different types of chronic liver diseases.
   1.4 To explore the role of Fn14 in AH and its correlations with important clinical features of the disease.
   1.5 To investigate the mechanisms leading to Fn14 up-regulation in AH.

2. **To unveil the role of CCL20 in liver injury and its potential role as driver of inflammation and fibrosis in AH.**

   2.1 To explore the hepatic and serum levels of CCL20 in patients with AH and other liver diseases.
   2.2 To assess the correlation of CCL20 expression with key clinical features of AH.
   2.3 To investigate the hepatic cell source of CCL20.
   2.4 To investigate the effects of CCL20 on human hepatic stellate cells.
   2.5 To explore the effects of CCL20 in animal models of liver injury.

RESULTS

FIRST ARTICLE

"Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis."

Affò S. et al. Gut 2013 Mar;62(3):452-60. Epub 2012 May 25

Alcoholic hepatitis (AH) is a severe clinical condition that needs urgently novel therapies. The identification of new potential targets for the treatment of this disease is hampered by the lack of well described animal models reproducing all the features of advanced AH. In this translational study we identified a profile of genes and pathways that are differentially regulated in patients with AH by performing a transcriptome analysis using liver samples from biopsies of patients with severe AH.

Transcriptome analysis identifies key pathways differentially regulated in AH

Using an original cohort of 40 patients with clinical, analytical and histological characteristics of AH, we selected a representative subgroup of 15 patients with severe AH to assesses a gene expression profile analysis. Patients included showed clinical, analytical and histological characteristics of AH. The majority of patients had severe sinusoidal portal hypertension, 78% of them were classified as severe AH (ABIC score >6.71) and had moderate or severe steatosis (69%) of diffuse distribution, marked hepatocyte ballooning (61%) and marked necro-inflammation (37%).

The transcriptome analysis revealed 207 genes >5-fold differentially regulated in patients with AH compared with controls. The most up-regulated genes that were differentially regulated in patients with severe AH included members of several pathways such as extracellular matrix (e.g. osteopontin, lysyl oxidase-like 4), inflammation (e.g. Fn14, CCL20) and cell cycle (Keratin -7-19-23) among others. Unsupervised clustering analysis showed a clear differentiation in gene expression between livers with AH and controls ($p<0.001$). All patients with AH clustered together and showed a homogeneous pattern of gene expression. Functional analysis was performed by gene set enrichment analysis and the Kyoto Encyclopedia of Genes and Genomes pathway database and revealed seven pathways differentially regulated in patients with AH including the cytokine–cytokine receptor

interaction and cell cycle and focal-adhesion pathways. The full microarray data have been deposited in NCBI's Gene Expression Omnibus (accession number GSE28619).

Cytokine–cytokine receptor interaction pathway is differentially regulated in chronic liver diseases

Cytokine-cytokine receptor pathway was one of the pathway with the higher number of deregulated genes in patients with AH compared with controls, and because cytokines are considered potential targets for therapy in AH, we investigated in more detail the cytokine–cytokine receptor interaction pathway. The microarray data confirmed previous results from our group showing that CXC chemokines (including CXCL4, IL-8, CXCL1) are markedly up-regulated in patients with AH. We also found that some CC chemokines including MCP-1 and CCL20 were also up-regulated in these patients. Interestingly, none of the agonists belonging to the TNF superfamily (including TNF-α and FasL) were differently expressed in livers of patients with AH. In contrast, several TNF superfamily receptors (e.g. FAS, TRAILR1 and Fn14) were markedly up-regulated.

In order to confirm the results obtained in the microarray analysis, we measured the hepatic expression of selected genes by quantitative PCR in our series of 40 patients with AH and in patients with other liver diseases including chronic hepatitis C (n=18) and NASH (n=20). Validated genes included seven CXC and two CC chemokines and genes belonging to the TNF superfamily (Fas, Fn14, TNF-α, TNFRSF1 and TRAILR1). The expression of selected genes assessed by real time PCR confirmed the results obtained with the microarray studies. Within all the TNF superfamily receptor validated, the only receptor exclusively up-regulated in AH, was Fn14. Fn14 was nearly 20-fold up-regulated in patients with AH, down-regulated in chronic hepatitis C and unchanged in NASH, suggesting that it may play a specific role in AH.

Moreover, we confirmed that TNF-α, FAS and TNFRSF1 are not increased in our series of patients with AH compared with normal livers. While TNF-α was increased in chronic hepatitis C ($p=0.0002$) and NASH ($p=0.02$), TNFRSF1 and FAS were exclusively up-regulated in patients with NASH ($p<0.0005$). Finally, we measured the expression of TRAF consensus binding motif contained in the cytoplasmic tail of most of the TNFR superfamily members including Fn14. We found that TRAF3, but not TRAF1, was increased in AH as compared to control samples.

Fn14 expression correlates with clinical features of AH and short-term mortality

Once identified Fn14 as the only TNF superfamily receptor exclusively up-regulated in patients with AH, we next explored the correlation between Fn14 hepatic expression and key clinical events that occur in patients with this liver disease. We found that hepatic expression of Fn14 correlated with short-term survival in patients with AH. Cox regression analysis showed that hepatic gene expression of Fn14 (HR: 1.05, 95% CI 1.00 to 1.11, p=0.03), IL-8 (HR: 1.14, 95% CI 1.02 to 1.26, p=0.019), CXCL5 (HR: 1.01, 95% CI 1.004 to 1.02, p=0.006), CXCL1 (HR: 1.001, 95% 1.00 to 1.003, p=0.018) and CXCL6 (HR: 1.01, 95% CI 1.004 to 1.03, p=0.009) was associated with 90-day mortality and Kaplan–Meier survival analysis was performed to compare 90-day mortality according to hepatic expression of Fn14 in patients with AH. Patients with higher Fn14 gene expression (>22 folds) had worse 90-day survival than patients with lower hepatic gene expression (p=0.02).

We next explored whether Fn14 expression correlated with other clinical features of AH such as the degree of portal hypertension, a major determinant of mortality in patients with AH. We found that patients with higher (>22-fold-expression) Fn14 gene expression showed more severe portal hypertension than patients with lower expression (<22-fold-expression) (HVPG 21.5±2 vs 17.5±1 mm Hg, respectively; p=0.04). Importantly, Fn14 and TRAILR1 hepatic gene expression correlated with the ABIC score (r=0.44, p=0.01 and r=0.41, p=0.02, respectively). In addition, hepatic gene expression of Fn14 positively correlated with most of CXC chemokines gene expression including CXCL1 and IL-8.

Fn14 co-localize with EpCAM positive hepatocytes in patients with AH

To investigate the cell source of Fn14 and its localization in the liver, we next investigated Fn14 at the protein level by immunohistochemistry. Fn14 staining was barely detected in normal human livers while it was mainly expressed in parenchymal cells around the fibrogenic areas in patients with AH. Because Fn14 has been reported to be expressed in progenitor cells in damaged tissues, we next explored whether Fn14 was expressed in progenitor cells or in hepatocytes that derive from these cells. For this reason, sequential liver sections from patients with AH were stained with anti-Fn14, anti-pan-cytokeratin, a marker expressed in progenitor cells; and anti-EpCAM, a surface marker of human hepatic progenitor cells that has been described to be also expressed in newly generated hepatocytes derived

from progenitor cells but not in mature hepatocytes. Interestingly, we found that Fn14 was expressed in hepatocytes at the edge of regenerative nodules co-localizing with EpCAM positive hepatocytes and that it was expressed only in a subpopulation of pan-cytokeratin positive cells.

Fn14 is up-regulated in experimental models of liver injury and is induced by TGF-β in precision-cut liver slices

Due to the lack of animal models reproducing all the features of human severe AH, we next explored the expression of Fn14 in mouse models of acute, chronic and acute-on-chronic liver injury. Fn14 hepatic gene expression was found increased in mice treated with CCl_4, DDC diet and acute acetaminophen ($p<0.05$). Otherwise, chronic administration of CCl_4 did not induce Fn14. Remarkably, we saw that the up-regulation of Fn14 was higher in the acetaminophen and DDC treated animals, two animal models of liver injury that are characterized by the expansion of liver progenitor cells. We also confirmed by immunohistochemistry that Fn14 was expressed in progenitor cells and weakly expressed in a subpopulation of hepatocytes. These results are in accordance with previous studies describing the expression of Fn14 in liver progenitor cells.

We next investigated the expression of Fn14 in an experimental model of acute and acute-on-chronic alcohol-induced liver damage. Acute ethanol exposure strongly induced Fn14 gene expression but did not induce other TNF-α superfamily members including TNF-α, TRAIL or TWEAK. Acute CCl_4 also induced an increase of hepatic Fn14, although to a lesser extent compared to ethanol. Nevertheless, chronic administration of CCl_4 did not induce an increase in Fn14 gene expression. Surprisingly, when we combined chronic administration of CCl_4 to a binge of ethanol, Fn14 was increased, though to a lesser extent than in the purely ethanol acute model.

We finally used high precision-cut liver slices from mice livers to investigate which mediators are involved in Fn14 up-regulation in the injured liver. Liver slices were exposed to LPS and TNF-α, two of the main inflammatory mediators involved in the pathogenesis of AH, as well as TWEAK (Fn14 ligand) and TGF-β1, a major fibrogenic mediator. Interestingly, we observed that the incubation of liver slices with TGF-β1 increased Fn14 hepatic gene expression, while LPS, TNFα or TWEAK did not induce its expression.

ORIGINAL ARTICLE

Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis

Silvia Affò,[1] Marlene Dominguez,[1] Juan José Lozano,[1] Pau Sancho-Bru,[1] Daniel Rodrigo-Torres,[1] Oriol Morales-Ibanez,[1] Montserrat Moreno,[1] Cristina Millán,[1] Aurora Loaeza-del-Castillo,[1] José Altamirano,[1] Juan Carlos García-Pagán,[1] Vicente Arroyo,[1] Pere Ginès,[1] Juan Caballería,[1] Robert F Schwabe,[2] Ramon Bataller[1]

► Additional materials are published online only. To view these files please visit the journal online (http://dx.doi.org/10.1136/gutjnl-2011-301146).

[1]Liver Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Centre Esther Koplowitz, CIBER de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain
[2]Department of Medicine, Columbia University, College of Physicians and Surgeons, New York, New York, USA

Correspondence to
Dr Ramon Bataller, Liver Unit, Hospital Clínic, Villarroel 170, Barcelona 08036, Spain; bataller@clinic.ub.es

Transcript profiling: Microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO; accession number GSE28619).

Revised 16 March 2012
Accepted 5 April 2012
Published Online First 25 May 2012

ABSTRACT

Objective Alcoholic hepatitis (AH) is a severe clinical condition that needs novel therapies. The identification of targets for therapy is hampered by the lack of animal models of advanced AH. The authors performed a translational study through a transcriptome analysis in patients with AH to identify new molecular targets.

Design Hepatic gene expression profiling was assessed by DNA microarray in patients with AH (n=15) and normal livers (n=7). Functional analysis was assessed by gene set enrichment analysis. Quantitative PCR was performed in patients with AH (n=40), hepatitis C (n=18), non-alcoholic steatohepatitis (n=20) and in mouse models of acute and chronic liver injury. Protein expression was assessed by immunohistochemistry and western blotting.

Results Gene expression analysis showed 207 genes >5-fold differentially expressed in patients with AH and revealed seven pathways differentially regulated including 'cytokine–cytokine receptor interaction'. Several tumour necrosis factor (TNF) superfamily receptors, but not ligands, were overexpressed in AH. Importantly, Fn14 was the only TNF superfamily receptor exclusively upregulated in AH compared with other liver diseases and correlated with both 90-day mortality and severity of portal hypertension. Fn14 protein expression was detected in areas of fibrogenesis and in a population of hepatocytes. Fn14 expression was increased in experimental models of liver injury and was detected in progenitor cells.

Conclusion Translational research revealed that TNF superfamily receptors are overexpressed in AH. Fn14, the receptor for TNF-like weak inducer of apoptosis, is selectively upregulated in patients with AH. TNF superfamily receptors could represent a potential target for therapy.

Significance of this study

What is already known about the subject?

- Alcoholic hepatitis (AH) is a severe form of alcoholic liver diseases that carries a poor short-term prognosis.
- Current therapies to treat AH (eg, corticosteroids) are not fully effective in many patients and targeted therapies are urgently needed.
- CXC chemokines are overexpressed in livers from patients with AH and may represent novel targets for therapy.
- Tumour necrosis factor α is overexpressed in animal models of moderate alcoholic liver disease.

What are the new findings?

- There are seven pathways differentially regulated in patients with alcoholic hepatitis (AH) compared with normal livers including 'focal adhesion', 'cell cycle' and 'cytokine–cytokine receptor interaction'.
- Tumour necrosis factor (TNF) superfamily receptors, but not ligands, are markedly overexpressed in patients with AH.
- Fn14 is the only TNF superfamily receptor exclusively upregulated in AH compared with other liver diseases and correlates with both 90-day mortality and severity of portal hypertension.

How might it impact on clinical practice in the foreseeable future?

- The identification of potential novel targets for therapy will stimulate the development of new targeted therapies for this severe clinical condition and will help the design of new clinical trials.

INTRODUCTION

Alcoholic hepatitis (AH) is the most severe form of alcoholic liver disease (ALD).[1] It is characterised by hepatocellular damage, steatosis and pericellular fibrosis. Patients with severe AH have a poor short-term prognosis.[2] Current therapies are not fully effective and novel targeted therapies are needed. The development of such therapies is hampered by a poor knowledge of the molecular mechanisms. Based on animal models,[3–6] tumour necrosis factor (TNF)α was proposed to play a pivotal role in AH.[3–9] Consequently, drugs interfering TNFα were tested in these patients[10 11] but the results were disappointing due to an increased incidence of severe bacterial infections.[12] There are no experimental models that mimic the main findings of AH

in humans. To overcome this limitation, translational studies with human samples are required.[13]

Microarray analysis using high-throughput screening technology has emerged as an important tool to study gene expression patterns and molecular events in complex diseases.[14] Functional interpretation of microarray data is currently performed using different softwares.[15] Gene profiling analysis has been performed in different types of chronic liver diseases.[16–18] Here, we use modern informatics tools to perform a functional analysis capable of identifying the pathways implicated in the pathogenesis of AH. We studied a series of biopsy-proven AH and fragments of normal livers and identified different pathways that may play a pathogenic role, including 'cytokine–cytokine receptor interaction'. A detailed analysis allowed us the identification of the TNF superfamily as a potential disease driver. The TNF superfamily contains a variety of cytokines and receptors that activate signalling pathways regulating cell survival, death and differentiation. The most relevant agonists include TNFα, TNF-like weak inducer of apoptosis (TWEAK) and Fas ligand. Studies from experimental models of alcoholic liver injury have implicated TNFα, Fas ligand and their receptors in liver damage and remodelling.[4 5 19 20] Little is known on the role of TWEAK and its specific receptor Fn14. Activation of Fn14 controls many cellular activities including proliferation, migration, differentiation, apoptosis, angiogenesis and inflammation.[21] Moreover, experimental evidence indicates that the TWEAK–Fn14 axis is implicated in progenitor cell expansion and liver regeneration.[22 23]

MATERIALS AND METHODS

Patients

Patients admitted to the Liver Unit of the Hospital Clínic, Barcelona (2007–2009), with clinical, analytical and histological features of AH were prospectively included. The inclusion criteria have been previously described.[13 24 25] All patients had histological diagnosis of AH. Patients with malignancies or any other potential cause of liver disease were excluded from the study. Liver biopsies were obtained using a transjugular approach. As controls, we included patients with chronic hepatitis C-induced liver disease (HCV) (n=18). All patients had HCV genotype 1 and did not receive previous antiviral treatment. We also included a cohort of patients with morbid obesity and associated non-alcoholic steatohepatitis (NASH) (n=20) according to Kleiner's criteria (supplementary table 1).[26] A laparoscopic liver biopsy was obtained in these patients during bariatric surgery. In all patients, liver specimens were analysed by an expert liver pathologist and a part of the biopsy was submerged into a RNA stabilisation solution (RNAlater, Ambion, Austin, Texas, USA). The protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Clinic of Barcelona and only patients with signed informed consent were included.

Selection of fragments of normal human livers

Fragments of normal livers were obtained from optimal cadaveric liver donors (n=3) or resection of liver metastases (n=4). All controls had normal serum aminotransferase levels and normal liver histology (supplementary table 2). Criteria to obtain normal livers were: (1) no past history of liver disease, alcohol abuse or metabolic syndrome; (2) absence of maintained arterial hypotension before laparotomy; (3) normal serum levels of aminotransferases; (4) time of brain death to laparotomy less than 12 h; (5) normal liver histology; (6) no use of vasodilator drugs; and (7) liver specimens obtained immediately after laparotomy and before vascular clamp. Samples obtained during resection of liver metastasis were obtained at a minimum distance of 5 cm from the tumour.

Microarray studies and functional analysis of microarray data

A subset of patients with severe AH based on clinical criteria (Maddrey's discriminant function >32) (n=15) was randomly selected to perform DNA microarray analysis. The epidemiological, clinical and analytical characteristics of this subset of patients were representative of the whole series of patients (n=40, table 1). High quality RNA samples were hybridised to GeneChips (Affymetrix *Hgu133plus*, Affymetrix, Santa Clara, California, USA) and a functional analysis was performed with the resulting data (see details in supplementary Materials and methods).

Real-time PCR

Quantitative real-time PCR (qPCR) was performed as previously described using commercial primer-probe pairs (Applied Biosystems, Foster City, California, USA) for CXCL3, CXCL4, CXCL5, CXCL6, CXCL10, Fas, TNFRSF12A (Fn14), CXCL1 (Gro-α), IL-8, CCL2 (MCP-1), osteoprotegerin, CCL5, TNFα, TNFRSF1, TRAF1, TRAF3, TRAILR1 and TNFSF12 (TWEAK).[27] Data were normalised to 18s and gene expression values were calculated based on the ΔΔCt method. The results were expressed as $2^{-\Delta\Delta Ct}$.

Immunohistochemistry

Paraffin-embedded liver sections were incubated with anti-Fn14 (1:40 overnight at 4°C, Abcam, Cambridge, UK), anti-epithelial cell adhesion molecule (EpCAM) (1:100 for 2 h at room temperature, Dako, Glostrup, Denmark), anti-pan-cytokeratin (1:4000 for 1 h at room temperature, Dako) and NF-κB p65 (1:50 overnight at 4°C, Cell Signalling, Beverly, Massachusetts, USA) primary antibodies.

Table 1 Clinical, analytical and hepatic haemodynamic characteristics of patients with alcoholic hepatitis (AH, n=40)

Variables	Mean±SE or percentage
Age (years)	49±1
Male (%)	65
Maddrey's discriminant function	59±6
MELD score	22±1
ABIC score	7.65±0.19
Alcohol intake (gr/d)	107±5
90-day mortality (%)	28
Glucose (mg/dl)	111±5
Creatinine (mg/dl)	0.84±0.06
AST (U/L)	155±12
ALT (U/L)	61±5
GGT (U/L)	600±83
Bilirubin (mg/dl)	12.5±1.21
Albumin (mg/dl)	26.2±0.55
Platelet count ($\times 10^9$)	139±14
Leucocytes count ($\times 10^9$/l)	10.04±7.43
Neutrophils count (%)	74±8.32
INR	1.92±0.12
HVPG (mm Hg)	19.5±1
Cirrhosis (%)	60
Severe AH (%)*	77.5

*Severe AH was defined as an ABIC score ≥6.71 points.
MELD, Model for End-stage Liver Disease; ABIC, Age-Bilirubin-INR-Creatinine score; ALT, alanine aminotransferase; AST, aspartate aminotransferse; GGT, gamma glutamyl transpeptidase; INR, international normalised rate; HVPG, hepatic venous pressure gradient.

Western blot

Western blot studies were performed using standard procedures. Membranes were incubated with antibodies against AKT, phospho-AKT (Ser473), p38 MAP Kinase, phospho-p38 MAPK (Thr180/Tyr182) (Cell Signaling) and reprobed with GAPDH (Abcam). After washes, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were detected by enhanced chemiluminescence (Biological Industries, Beit-Haemek, Israel).

High precision-cut liver slices of mice livers

Male C57BL/6 mice were used to obtain 250 μm slices from fresh liver sections using a Vibratome VT1000S (Leica Microsystems, Wetzlar, Germany). Samples were washed in PBS, soaked in 4% agarose solution (Ultrapure LMP Agarose, Invitrogen, Carlsbad, California, USA) for 20 min, and then orientated, mounted and immobilised using cyanoacrylate glue. Tissue slices were placed on organotypic tissue culture plate inserts for up to 24 h (Millicell®-CM; Millipore, Massachusetts, USA). Tissues were maintained at 37°C in a 5% CO_2 humidified incubator using 1.1 ml of Williams'Medium E (Sigma-Aldrich, St. Louis, Missouri, USA) supplemented with 1% inactivated fetal bovine serum, 2 mM L-Glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 μM insulin, 15 mM HEPES and 50 μM β-mercaptoetanol for up to 48 h. After 24 h in culture, tissue slices were incubated with TWEAK (R&D Systems, Minneapolis, Minnesota, USA), TGFβ1 (Sigma-Aldrich), TNFα (R&D Systems) and lipopolysaccharide (LPS, Sigma-Aldrich) for 24 h. Slices were then transferred to a 1.5 ml tube and homogenised with a polypropylene pestle in 1 ml Trizol®Reagent and total RNA was obtained.

Experimental mouse models

Different models of acute and chronic liver injury were performed including carbon tetrachloride (CCl_4), 3,5-diethoxycarbonyl-1,4-dihydro-collidin (DDC) and acetaminophen administration (see supplementary Materials and methods for details). Moreover, we used a model of acute and acute-on-chronic ethanol-induced liver injury. In the acute model, male Balb/c mice (n=10) were fasted for 8 h with free access to water and then gavaged a single dose of 50% ethanol (5 g/kg body weight) or water. Animals were sacrificed 8 h after gavage. In the acute-on-chronic model, male Balb/c mice (n=10) were intraperitoneally administered with CCl_4 (Sigma-Aldrich; diluted 1:4 in oil) or vehicle (oil) at a dose of 0.5 ml/kg body weight twice per week for a total of five injections. Two days after the last CCl_4 injection, mice were fasted for 8 h with free access to water, and then they were gavaged a single dose of 50% ethanol (5 g/kg body weight) or water and sacrificed 8 h after gavage.

In all animal models, livers were excised and collected for RNA extraction and immunohistochemistry. All animal procedures were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Statistical analysis

Continuous variables were described as means (±SE) and were compared using Student t test or Mann–Whitney U test, as appropriate, depending on their normality test. Differences between categorical variables were assessed by Fisher's exact test or the χ^2 test with Yates correction for continuity, when necessary.

Correlations between variables were evaluated using Spearman's ρ or Pearson's r, when appropriate. In order to identify those molecules associated with short-term mortality (90 days) we fitted a Cox univariate regression analysis. We did not perform a multivariate Cox regression analysis because of the high risk of overfitting due to the scarce numbers of events (n=11) in our sample, according to a proportion rule of variables/events of 1:10.

The area under the receiver characteristic curve (AUROC) analysis was used to determine the best cut-off value and the accuracy (sensitivity and specificity) of continuous variables associated with 90-day mortality. Finally, we performed a comparative risk analysis using the Kaplan–Meier method. Comparisons were performed by the log-rank test. All statistical analyses were performed using SPSS V.14.0 for Windows (SPSS Inc.).

RESULTS

General characteristics of patients

We prospectively included 40 patients with clinical, analytical and histological characteristics of AH. Thirty patients (75%) developed at least one major complication during hospitalisation: two-thirds presented ascites and almost a half of the patients developed bacterial infections, whereas only a minority developed renal failure, encephalopathy or variceal haemorrhage.

The majority of patients had severe sinusoidal portal hypertension and 78% were classified as severe AH (ABIC score >6.71) at admission (for ABIC score calculation see: http://www.lille-model.com/score.asp?score=abic).[25] The majority of patients had moderate or severe steatosis (69%) of diffuse distribution, marked hepatocyte ballooning (61%) and marked necro-inflammation (37%). The main general characteristics of patients are depicted in table 1.

Unsupervised hierarchical clustering analysis

Gene expression profile analysis was first assessed in a representative subgroup of patients with severe AH (n=15) that was obtained from the original cohort (n=40). This analysis revealed 207 genes >5-fold differentially regulated in patients with AH compared with controls. The most upregulated genes that were differentially regulated are shown in table 2. Unsupervised clustering analysis allowed a clear differentiation in gene expression between livers with AH and controls ($p<0.001$) (figure 1). All patients with AH clustered together and showed a homogeneous pattern of gene expression. Functional analysis assessed using the gene set enrichment analysis program and the Kyoto Encyclopedia of Genes and Genomes pathway database revealed seven pathways differentially regulated in patients with AH including the cytokine–cytokine receptor interaction pathway (table 3). Full microarray data have been deposited in NCBI's Gene Expression Omnibus (accession number GSE28619).

Analysis of the cytokine–cytokine receptor interaction pathway in different types of chronic liver diseases

Because cytokines are currently considered potential targets of therapy in patients with AH, we focused on the cytokine–cytokine receptor interaction pathway (figure 2). First, the microarray data confirmed previous results from our group indicating that CXC chemokines are markedly overexpressed in AH.[13 24] Second, we found that some CC chemokines including MCP-1 were upregulated in these patients while others remained unchanged. Interestingly, none of the agonists belonging to the TNF superfamily (including TNFα and Fas ligand) were differently expressed in livers with AH. In contrast, several TNF

Table 2 Most relevant genes differentially expressed in patients with alcoholic hepatitis compared with controls

Accession N°	Gene name	Fold expression
	Extracellular matrix/fibrosis	
M 83248	Osteopontin	67.17
AW190565	Lysyl oxidase-like 4	39.95
NM_000089	Collagen, type I, α 2	38.23
NM_005764	PDZK1 interacting protein 1	35.08
NM_002423	Matrix metallopeptidase 7	28.70
NM_003247	Thrombospondin 2	16.69
K01228	Collagen, type I, α 1	14.97
N30339	Collagen, type V, α 1	14.49
BC001388	Annexin A2	12.72
NM_001845	Collagen, type IV, α 1	10.54
NM_003254	TIMP metallopeptidase inhibitor 1	8.88
AU146808	Collagen, type III, α 1	8.76
X05610	Collagen, type IV, α 2	7.65
AK026829	Laminin, α 2	6.70
NM_000393	Collagen, type V, α 2	6.18
	Inflammation/immunity	
NM_004591	Chemokine (C-C motif) ligand 20	90.21
NM_002993	Chemokine (C-X-C motif) ligand 6	45.70
NM_004221	Interleukin 32	15.37
NM_000584	Interleukin 8	14.90
NM_016639	Tumour necrosis factor receptor superfamily, member 12A (Fn14)	10.32
NM_001511	Chemokine CXC ligand 1 (Gro-α)	9.42
NM_001565	Chemokine CXC ligand 10	6.99
AI817041	Chemokine CXC receptor 7	5.32
	Cell communication/cell cycle/apoptosis	
NM_015515	Keratin 23 (histone deacetylase inducible)	126.81
BG327863	CD24 molecule	21.29
BC002700	Keratin 7	15.26
NM_002276	Keratin 19	6.41
NM_000224	Keratin 18	5.06
	Other functions	
NM_020299	Aldo-keto reductase family 1, member B10	484.19
NM_005564	Lipocalin 2	56.66
NM_006398	Ubiquitin D	56.64
NM_016548	Golgi membrane protein 1	31.50
J04152	Tumour-associated calcium signal transducer 2	22.40
NM_002354	Tumour-associated calcium signal transducer 1	17.49
NM_000903	NAD(P)H dehydrogenase, quinone 1	16.91
AL514445	Regulator of G-protein signalling 4	15.65
NM_001442	Fatty acid binding protein 4, adipocyte	15.32
NM_005980	S100 calcium binding protein P	10.50

superfamily receptors (eg, FAS, TRAILR1 and Fn14) were markedly overexpressed. To confirm the results obtained in the microarray analysis, we measured the expression of selected genes by quantitative PCR in our series of patients with AH (n=40) and in patients with other liver diseases including chronic hepatitis C (n=18) and NASH (n=20). Validated genes included seven CXC and two CC chemokines and genes belonging to the TNF superfamily (Fas, Fn14, TNFα, TNFRSF1 and TRAILR1). The expression of the selected genes assessed by qPCR corroborated the results obtained with the microarray studies ($p<0.01$ for all). Among TNF receptor superfamily, both Fn14 and TRAILR1 were markedly overexpressed in AH compared with normal livers ($p<0.001$) (figure 3A). Importantly, the only TNF superfamily receptor exclusively overexpressed in AH was Fn14. Fn14 was nearly 20-fold overexpressed in AH, downregulated in chronic hepatitis C and unchanged in NASH,

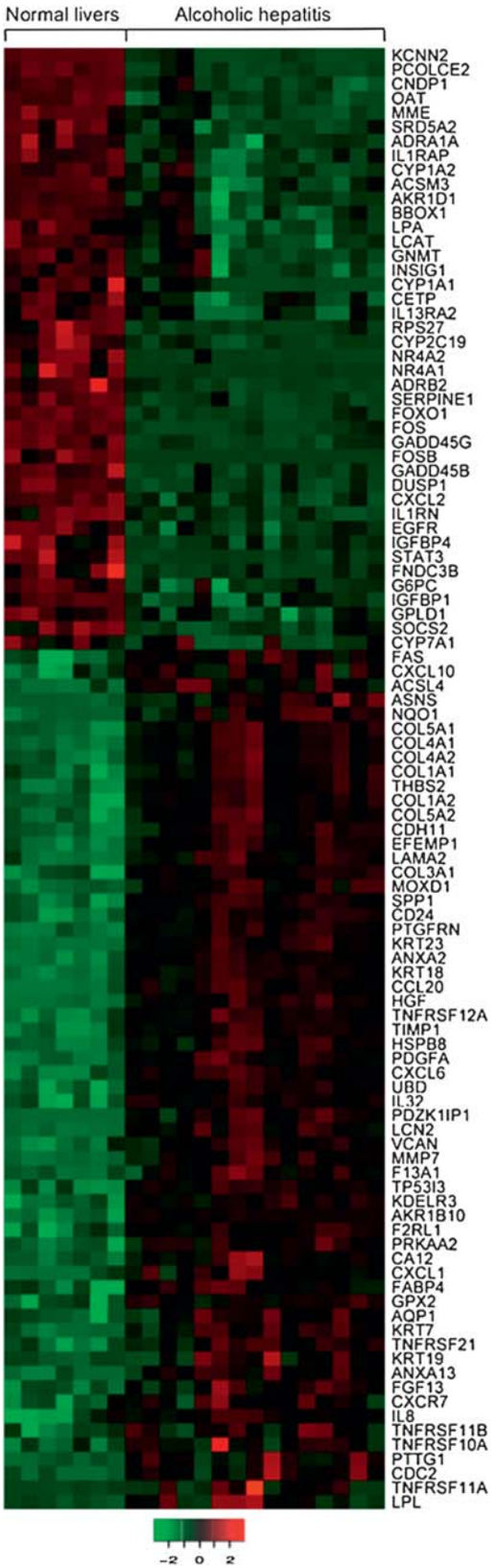


Figure 1 Microarray data of patients with alcoholic hepatitis (n=15) compared with control livers (n=7). The intensity of each colour denotes the standardised ratio between each value and the average expression of each gene across all samples. Red coloured pixels correspond to an increased abundance of the transcript in the indicated sample, whereas green pixels indicate decreased transcript levels.

Table 3 Biological pathways for differentially expressed genes in patients with alcoholic hepatitis revealed in the functional analysis

Pathways	ID	Involved genes	FDR
Focal adhesion	HSA04510	58	0.03
Cell cycle	HSA04110	49	0.05
Cytokine–cytokine receptor interaction	HSA04060	33	0.05
Cell communication	HSA01430	24	0.01
Extracellular matrix–receptor interaction	HSA04512	24	0.01
Aminoacyl-tRNA biosynthesis	HSA00970	23	0.17
GAP junction	HSA04540	15	0.14

FDR, false discovery rates

suggesting that it may play a specific role in AH. Moreover, we confirmed that TNFα, FAS and TNFRSF1 are not increased in our series of patients with AH compared with normal livers. While TNFα was increased in chronic hepatitis C (p=0.0002) and NASH (p=0.02), TNFRSF1 and FAS were exclusively upregulated in patients with NASH (p<0.0005). Finally, we measured the expression of TNFR-associated factor (TRAF) consensus binding motif contained in the cytoplasmic tail of most of the TNFR superfamily members including Fn14.[21] We found that TRAF3, but not TRAF1, was increased in AH (figure 3A).

Relationship between Fn14 and short-term prognosis in patients with AH

Because Fn14 was the only TNF superfamily receptor exclusively overexpressed in AH (figure 3A), we next explored the correlation between Fn14 hepatic expression and key clinical events. We explored whether baseline hepatic expression of Fn14 correlates with short-term survival in patients with AH. Cox regression analysis showed that hepatic gene expression of Fn14 (HR: 1.05, 95% CI 1.00 to 1.11, p=0.03), IL-8 (HR: 1.14, 95% CI 1.02 to 1.26, p=0.019), CXCL-5 (HR: 1.01, 95% CI 1.004 to 1.02, p=0.006), CXCL1 (HR: 1.001, 95% 1.00 to 1.003, p=0.018) and CXCL6 (HR: 1.01, 95% CI 1.004 to 1.03, p=0.009) was associated with 90-day mortality. The best cut-off level for Fn14 gene expression was 22 ($2^{-\Delta\Delta Ct}$) (AUROC: 0.72; sensitivity 75% and specificity 62%, data not shown). Kaplan–Meier survival analysis was performed to compare 90-day mortality according to hepatic expression of Fn14 in patients with AH. Patients with higher Fn14 gene expression (>22) had worse 90-day survival than patients with lower hepatic gene expression (p=0.02) (figure 3B). We next explored whether Fn14 expression correlated with the degree of portal hypertension, a major determinant of mortality in patients with AH. Patients with high (>22-fold-expression) Fn14 gene expression showed more severe portal hypertension than patients with low expression (<22-fold-expression) (HVPG 21.5±2 vs 17.5±1 mm Hg, respectively; p=0.04) (figure 3C). Importantly, Fn14 and TRAILR1 hepatic gene expression correlated with the ABIC score, a system that identifies patients with severe disease (r=0.44, p=0.01 and r=0.41, p=0.02, respectively). In addition, hepatic gene expression of Fn14 positively correlated with most of CXC chemokines gene expression (supplementary figure 1). Collectively, these results suggest that Fn14 could be implicated in the pathogenesis of disease severity in AH.

Immunohistochemistry analysis of Fn14 expression in patients with AH

We next studied Fn14 at the protein level. Fn14 staining was barely detected in normal human livers while it was mainly

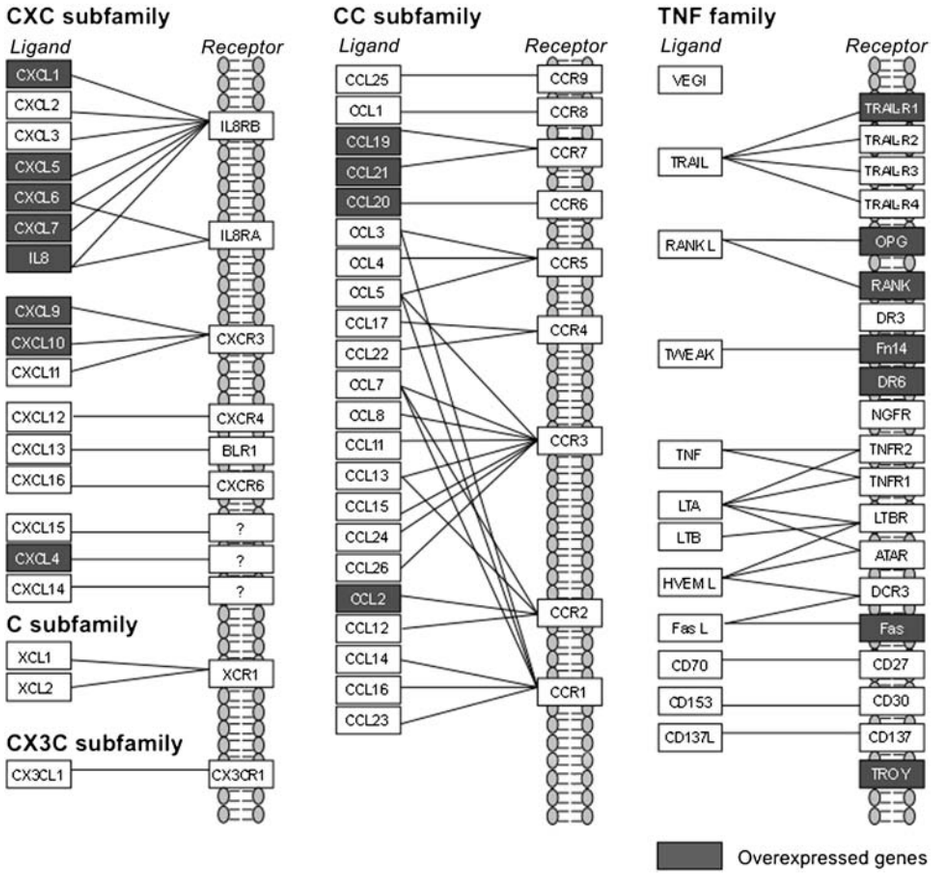


Figure 2 Gene expression of the members of 'cytokine–cytokine receptor interaction' pathway in patients with alcoholic hepatitis (AH). In grey colour, ligands and receptors that were found upregulated in patients with AH.

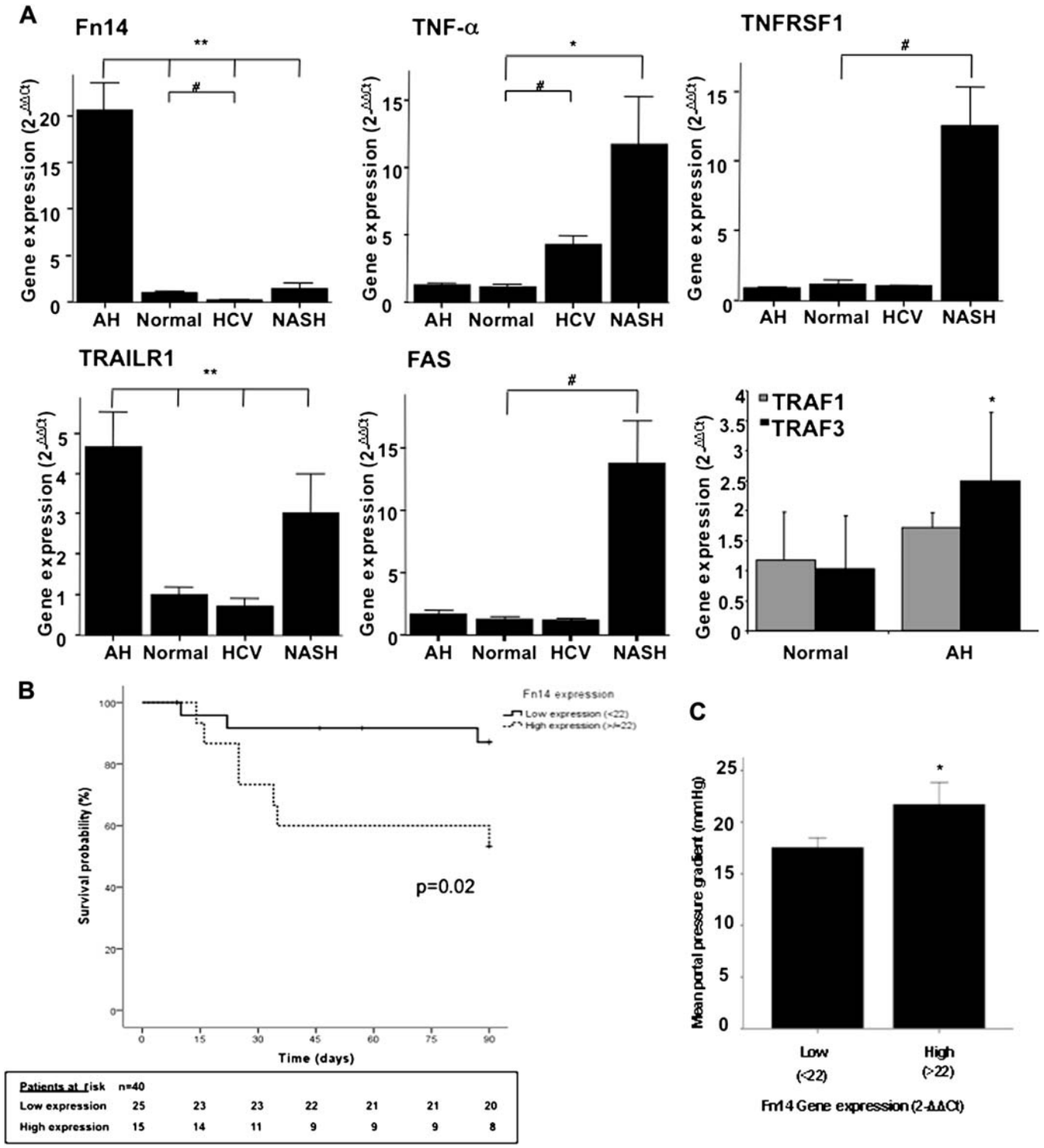


Figure 3 (A) Fn14, TNFα, TNFRSF1, TRAILR1 and FAS gene expression in patients with alcoholic hepatitis (AH) (n=40), HCV (n=18) and non-alcoholic steatohepatitis (NASH) (n=20) compared with normal livers (n=7) (*p<0.05; **p<0.005; #p<0.0005); TRAF1 and TRAF3 gene expression in patients with AH compared with normal livers (*p<0.05). (B) Kaplan–Meier curve showing 90-day survival according to Fn14 gene expression at baseline. A value of 22-fold expression with respect to controls was identified as the cut-off value with better sensitivity and specificity to define patients with low and high Fn14 gene expression. (C) Severity of portal hypertension among patients with low (<22-fold) and high (>22-fold) Fn14 gene expression (*p=0.04).

expressed in parenchymal cells around the fibrogenic areas in patients with AH (figure 4A,B). Because Fn14 has been reported to be expressed in progenitor cells in damaged tissues[22 23] we next explored whether Fn14 was expressed in progenitor cells or in hepatocytes that derive from these cells. For this purpose, sequential liver sections from patients with AH were stained with anti-Fn14, anti-pan-cytokeratin (a marker of progenitor cells) and anti-EpCAM, a surface marker of human hepatic progenitor cells that is also expressed in newly generated hepatocytes derived from progenitor cells but not in mature

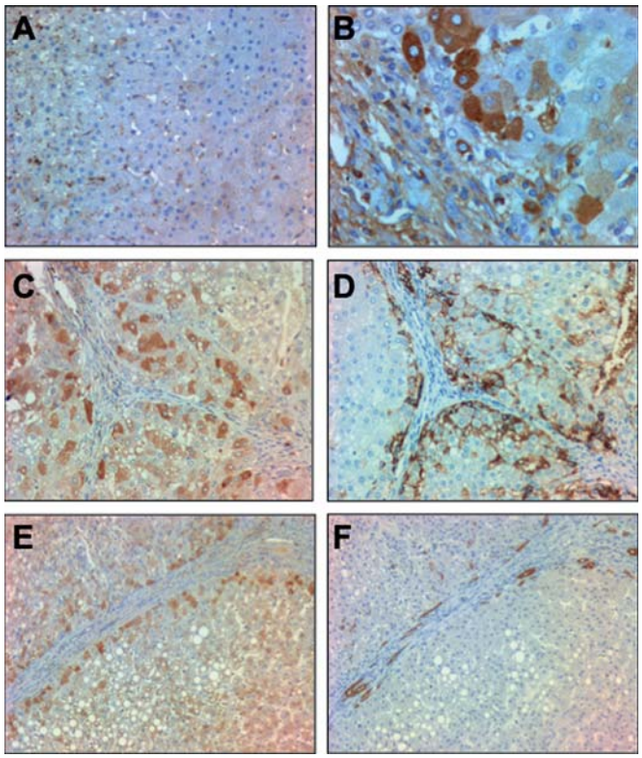


Figure 4 (A) Representative photomicrograph of a normal liver staining for Fn14 (200× magnification); (B) Representative photomicrograph of a liver with alcoholic hepatitis (AH) stained with anti-Fn14 (original magnification, ×400); (C, D) Immunohistochemistry of consecutive slices of livers with AH stained with anti-Fn14 and anti-EpCAM, respectively (original magnification, ×200); (E, F) Immunohistochemistry of consecutive slices of livers with AH stained with anti-Fn14 and anti-pan-cytokeratin antibodies, respectively (original magnification, ×200).

hepatocytes.[28] Fn14 was expressed in hepatocytes at the edge of regenerative nodules colocalising with EpCAM positive cells (figure 4C,D) and it was expressed only in a subpopulation of pan-cytokeratin positive cells (figure 4E,F). These results suggest that Fn14 is mainly expressed in a fraction of hepatocytes and in a subpopulation of progenitor cells in patients with AH.

Expression of Fn14 in experimental models and in precision-cut liver slices in mice

Because there are no animal models of severe AH, we next explored the expression of Fn14 in mouse models of acute and chronic liver injury. Well-established animal models of acute and chronic liver injury were performed. Fn14 gene expression ($p<0.05$) was increased in mice treated with CCl_4, DDC diet and acute acetaminophen, but not by chronic administration of CCl_4 (figure 5A). Remarkably, overexpression of Fn14 was higher in the acetaminophen and DDC treated animals, two animal models of liver injury that are characterised by the expansion of liver progenitor cells. Immunohistochemical studies showed that Fn14 was expressed in progenitor cells (figure 5B) and weakly expressed in a subpopulation of hepatocytes. These results are in accordance with previous studies describing the expression of Fn14 in liver progenitor cells.[22 23]

We next studied the expression of Fn14 in an experimental model of acute and acute-on-chronic alcohol-induced liver damage. Acute ethanol exposure strongly induced Fn14 gene expression (figure 5C) but not other TNFα superfamily members including TNFα, TRAIL and TWEAK (data not shown). In the acute-on-chronic model, Fn14 was also increased, but in a lesser extent than in the purely acute model (figure 5C). Moreover, we studied several signalling pathways potentially implicated in Fn14-induced biological effects. We found that p38, AKT and NF-κB p65 were activated in the livers of animals exposed to ethanol compared with control mice (figure 5D,E).

We finally used high precision-cut liver slices from mice livers to investigate which mediators are involved in Fn14 upregulation in the injured liver. Liver slices were exposed to inflammatory mediators involved in the pathogenesis of AH (LPS and TNFα) as well as TWEAK and TGFβ1, a major fibrogenic mediator. Incubation of liver slices with LPS, TNFα and TWEAK did not increase Fn14 gene expression, while TGFβ1 increased its expression (figure 5E).

DISCUSSION

The current study investigates the hepatic gene expression profile in patients with AH using a high-throughput approach. We performed a functional analysis of the hepatic transcriptome, which allowed us to identify several pathways potentially implicated in the pathogenesis of this devastating medical condition. Besides confirming some previous data[13 24] we provide evidence that other novel biological pathways such as 'TNF superfamily receptors' and 'ECM (extracellular matrix)—receptors interaction' are potentially implicated in this disease. The results of this study could be relevant, since it could provide useful human data to research groups devoted to the study of ALD. Importantly, some of the results obtained in our study differ from those obtained in rodents exposed to alcohol (ie, the lack of hepatic upregulation of TNFα and its specific receptors).[4 5] These discrepancies between human and experimental data reinforce the need to develop an experimental model that reproduces all the features of AH.

Because current therapies (ie, corticosteroids) are not effective in many patients with severe AH,[29 30] targeted therapies are urgently needed. In the absence of a well-accepted model of AH in rodents, studies in human samples seem mandatory. Our translational approach to identify novel therapies consists of analysing gene expression in the livers from patients with biopsy-proven AH. In all patients, liver specimens were obtained using a transjugular approach by an experienced team, which also allowed the measurement of portal pressure. We selected carefully the patients so it was not surprising that gene expression pattern was quite homogeneous throughout our series. It is important to stress that the study was performed in patients with the most severe form of ALD. Whether the results obtained in our study also apply to patients with moderate forms of ALD is unknown and deserves further investigation.

A functional analysis revealed a homogeneous pattern of altered hepatic gene expression in patients with AH. This pattern allowed the identification of several pathways implicated in AH including cytokine—cytokine receptor interaction. A detailed analysis of these pathways revealed relevant information. First, we confirmed previous data from our laboratory suggesting that CXC chemokines such as Gro-α and IL-8 may play a major role in AH.[13 24] Second, we provide evidence that some selected CC chemokines such MCP-1 are also differentially regulated in these patients. This finding could be relevant since we previously found a marked lymphocytic infiltration in livers with AH, which is the hallmark biological property of CC chemokines.[13] We are currently investigating the functional role of this type of chemokines in our laboratory. Most importantly, data obtained in the microarray analysis and quantitative PCR

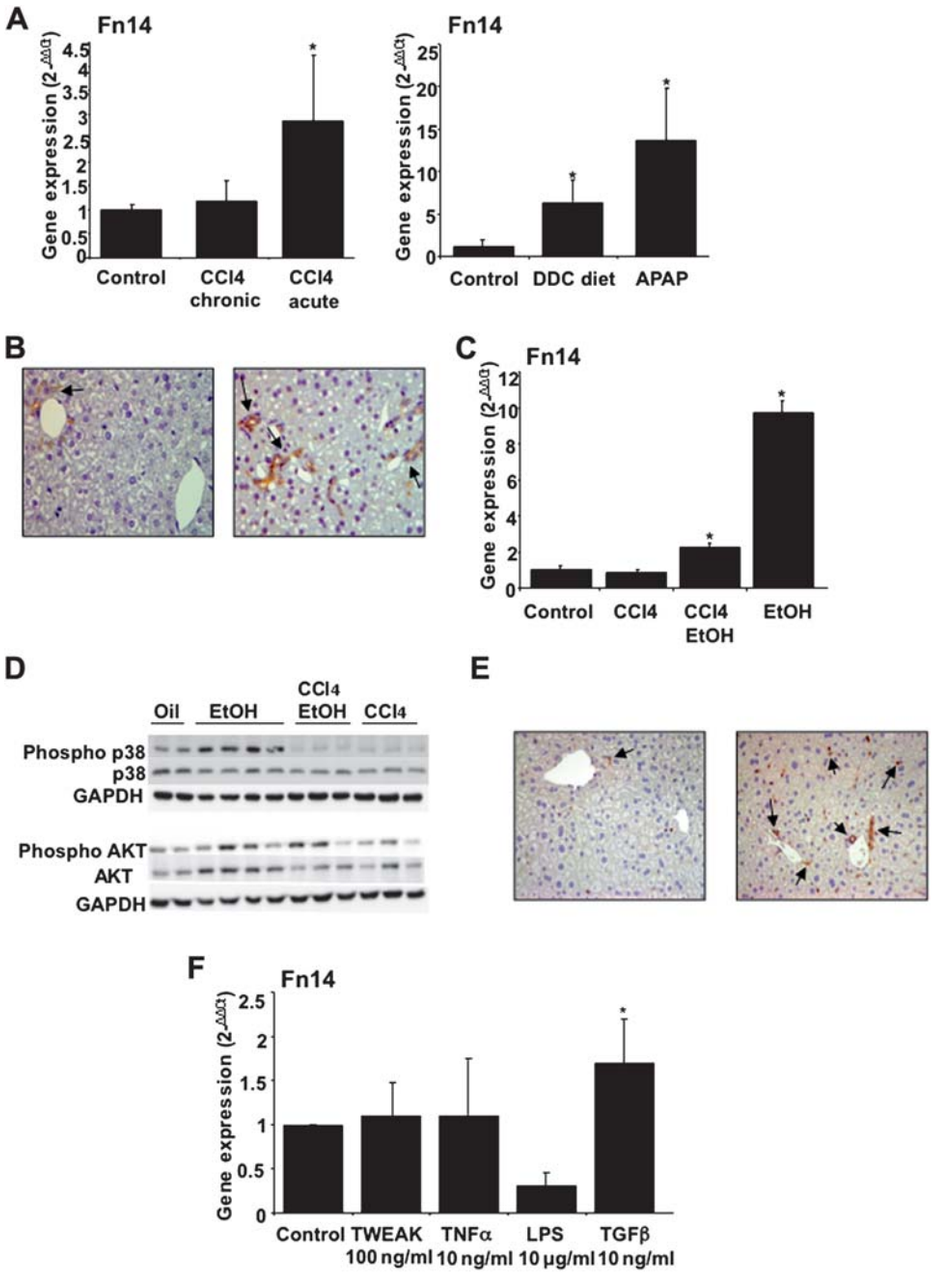


Figure 5 (A) Hepatic Fn14 gene expression in mice with chronic and acute CCl_4 administration and DDC diet treated and receiving acetaminophen (APAP) mice (see supplementary Materials and methods) (*$p<0.05$ with respect to controls); (B) Fn14 hepatic protein expression in control mice and in mice treated with APAP; Fn14 expression was detected in progenitor cells (arrows) and was weakly expressed in some hepatocytes (original magnification, ×400); (C) Hepatic Fn14 gene expression from mice with CCl_4 administration, CCl_4 plus binge ethanol (EtOH) gavage and binge EtOH gavage (see Materials and methods) (*$p<0.05$ with respect to controls); (D) Western blot analysis from the same groups of mice showed in (C). Representative western blot of phospho-p38 MAPK (Thr180/Tyr182), p38 MAP Kinase, phospho-AKT (Ser473) and AKT reprobed with GAPDH; (E) Representative immunohistochemical images of control and gavage EtOH administrated mice (200× magnification) using anti-NF-κB p65 antibody; NF-κB p65 was strongly activated in the liver of mice gavaged with EtOH in progenitor cells and in some hepatocytes as well as in inflammatory cells (arrows); (F) Fn14 hepatic gene expression in mice high precision-cut liver slices exposed to different mediators including TWEAK, TNFα, LPS and TGFβ1. TGFβ1 was the only mediator that increased Fn14 gene expression (*$p<0.05$).

studies corroborated previous results showing that TNFα is not differentially expressed in livers with AH. Therefore, we think that the current paradigm that TNFα plays a pivotal role in severe cases of ALD may be revised. These results could have important therapeutic implications, since they can explain, at least in part, why TNFα blocking agents are ineffective in these patients.[12]

The most striking finding of our study was the marked upregulation of several receptors belonging to the TNF superfamily such as Fn14, TRAILR1 and FAS in patients with AH. In contrast, none of the ligands including TWEAK, TRAIL, FAS ligand and TNFα were differentially regulated. Interestingly, we found that Fn14 was the only receptor exclusively overexpressed in patients with AH, while TRAILR1 was overexpressed in AH and NASH and TNFRSF1 and FAS were exclusively upregulated in NASH. Because of the specific overexpression of Fn14 in AH patients, we decided to further explore this TNF receptor. We first investigated whether Fn14 hepatic expression at admission predicts mortality in patients with AH. Fn14 expression was markedly associated with mortality rate at 3 months, suggesting a potential pathogenic role for this receptor in the pathogenesis of AH.

Fn14 is the receptor for TWEAK, a cytokine belonging to the TNF superfamily that has powerful biological properties.[31] Importantly, Fn14 is also able to signal in a ligand-independent manner when it is ectopically overexpressed in vitro.[21] We hypothesise that in AH, Fn14 could act in a TWEAK-independent manner. In fact, experimental studies in precision-cut liver slices from mice showed that TWEAK did not induce Fn14 gene expression even if capable of inducing other pro-fibrogenic and pro-inflammatory genes in the liver (supplementary figure 2). Moreover, TWEAK serum levels were not increased in patients with AH. The mechanisms leading to Fn14 upregulation in AH are actually unknown. We showed that TGFβ1, a profibrogenic cytokine markedly increased in AH, increases hepatic expression of Fn14. This effect was not induced by TNFα, TWEAK and LPS,

suggesting that fibrogenic, rather than inflammatory mediators, could regulate Fn14 hepatic expression.

Recent data from our laboratory indicate that liver regeneration is probably impaired in patients with AH.[27] Accumulation of hepatic progenitor cells correlates with patient mortality, suggesting a defect in the maturation process that generates functional hepatocytes in these patients. Because Fn14 is expressed in progenitor cells in damaged tissues,[22 23] we explored whether Fn14 is also expressed in experimental models characterised by acute and chronic liver damage and progenitor cell expansion. In both models, we found a marked expression of Fn14. This finding, together with the colocalisation of Fn14 with progenitor cells and newly generated hepatocytes derived from progenitor cells in livers from patients with AH, suggests a potential role for this receptor in progenitor cell differentiation. Importantly, ethanol administration to mice resulted in increased Fn14 expression, suggesting that alcohol abuse stimulates Fn14 expression in the liver. Functional studies modifying Fn14 expression in an animal model of AH are required to delineate the precise role of this pathway in AH.

In conclusion, our study demonstrates that several pathways, including the cytokine–cytokine receptor interaction, may play a role in AH in humans. Among genes belonging to this pathway, we found that TNFα superfamily receptors are markedly upregulated. One of these receptors, Fn14, was exclusively overexpressed in AH compared with other types of chronic liver diseases and to control livers. Fn14 hepatic expression correlated with disease severity and degree of portal hypertension. Future studies in animal models of acute AH are required to delineate the role of Fn14 in liver injury and regeneration in this severe liver disease.

Contributors SA and MD performed the experiments, collected and analysed data, interpreted the data and wrote the manuscript. JJL: analysed data and interpreted the data of the microarray. PS-B, DR-T, OM-I, MM, CM, ALC, JA: collected and analysed data, interpreted the data and contributed in the revision of the manuscript. JCG-P, VA, PG, JC, RFS: interpreted the data and contributed to the revision of the manuscript. RB: designed the study, interpreted the data and wrote the manuscript. SA and MD contributed equally to this work.

Funding This study was supported by grants from the Instituto de Salud Carlos III (FIS PI080237, FIS PS09/01164 and FIS PI080126 to RB, JC and PG, respectively). SA received a grant from IDIBAPS. PS-B was funded by the Ministerio de Ciencia e Innovación, Juan de la Cierva (JCI-2009-03849) and by the Instituto de Salud Carlos III, Miguel Servet (CP11/00071). DR-T received a grant from the Ministerio de Educación, FPU programme. MD and JA received a grant from Fundación Banco Bilbao Vizcaya Argentaria. This work was performed in Centre Esther Koplowitz (CEK).

Competing interests None.

Ethics approval Approval provided by the Ethics Committee of the Hospital Clinic of Barcelona, Spain.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1. **Nagata K,** Suzuki H, Sakaguchi S. Common pathogenic mechanism in development progression of liver injury caused by non-alcoholic or alcoholic steatohepatitis. *J Toxicol Sci* 2007;**32**:453–68.
2. **Lucey MR,** Mathurin P, Morgan TR. Alcoholic hepatitis. *N Engl J Med* 2009;**360**:2758–69.
3. **Tilg H,** Diehl AM. Cytokines in alcoholic and non alcoholic steatohepatitis. *N Engl J Med* 2000;**343**:1467–76.
4. **Iimuro Y,** Gallucci RM, Luster MI, *et al*. Antibodies to tumour necrosis factor alpha attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat. *Hepatology* 1997;**26**:1530–7.
5. **Yin M,** Wheeler MD, Kono H, *et al*. Essential role of tumour necrosis factor alpha in alcohol-induced liver injury in mice. *Gastroenterology* 1999;**117**:942–5.
6. **Neuman MG,** Brenner DA, Rehermann B, *et al*. Mechanisms of alcoholic liver disease: cytokines. *Alcohol Clin Exp Res* 2001;**25**(5 Suppl ISBRA):251S–3S.
7. **Mcclain CJ,** Barve S, Deaciuc I, *et al*. Tumor necrosis factor and alcoholic liver disease. *Alcohol Clin Exp Res* 1998;**22**:248S–52S.
8. **Mcclain CJ,** Barve S, Deaciuc I, *et al*. Cytokines in alcoholic liver disease. *Semin Liver Dis* 1999;**19**:205–19.
9. **Mookerjee RP,** Sen S, Davies NA, *et al*. Tumour necrosis factor alpha is an important mediator of portal and systemic haemodynamic derangements in alcoholic hepatitis. *Gut* 2003;**52**:1182–7.
10. **Spahr L,** Rubbia-Brandt L, Frossard JL, *et al*. Combination of steroids with infliximab or placebo in severe alcoholic hepatitis: a randomized controlled pilot study. *J Hepatol* 2002;**37**:448–55.
11. **Tilg H,** Jalan R, Kaser A, *et al*. Anti-tumor necrosis factor-alpha monoclonal antobody therapy in severe alcoholic hepatitis. *J Hepatol* 2003;**38**:419–25.
12. **Naveau S,** Chollet-Martin S, Dharancy S, *et al*. A double-blind randomized controlled trial of infliximab associated with prednisolone in acute alcoholic hepatitis. *Hepatology* 2004;**39**:1390–7.
13. **Dominguez M,** Miquel R, Colmenero J, *et al*. Hepatic expression of CXC chemokines predicts portal hypertension and survival in patients with alcoholic hepatitis. *Gastroenterology* 2009;**136**:1639–50.
14. **Fu S,** Zhao H, Shi J, *et al*. Peripheral arterial occlusive disease: global gene expression analyses suggest a major role for immune and inflammatory responses. *BMC Genomics* 2008;**9**:369.
15. **Dopazo J.** Functional interpretation of microarray experiments. *OMICS* 2006;**10**:398–410.
16. **Seth D,** Leo MA, McGuinness PH, *et al*. Gene expression profiling of alcoholic liver disease in the baboon (Papio hamadryas) and human liver. *Am J Pathol* 2003;**163**:2303–17.
17. **Seth D,** Gorrell MD, McGuinness PH, *et al*. SMART amplification maintains representation of relative gene expression: quantitative validation by real time PCR and application to studies of alcoholic liver disease in primates. *J Biochem Biophys Methods* 2003;**55**:53–66.
18. **Seth D,** Gorrell MD, Cordoba S, *et al*. Intrahepatic gene expression in human alcoholic hepatitis. *J Hepatol* 2006;**45**:306–20.
19. **Tagami A,** Ohnishi H, Moriwaki H, *et al*. Fas-mediated apoptosis in acute alcoholic hepatitis. *Hepatogastroenterology* 2003;**50**:443–8.
20. **Taïeb J,** Mathurin P, Poynard T, *et al*. Raised plasma soluble Fas and Fas-ligand in alcoholic liver disease. *Lancet* 1998;**351**:1930–1.
21. **Winkles JA.** The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting. *Nat Rev Drug Discov* 2008;**7**:411–25.
22. **Jakubowski A,** Ambrose C, Parr M, *et al*. TWEAK induces liver progenitor cell proliferation. *J Clin Invest* 2005;**115**:2330–40.
23. **Tirnitz-Parker JEE,** Viebahn SC, Jakubowski A, *et al*. Tumor necrosis factor–like weak inducer of apoptosis is a mitogen for liver progenitor cells. *Hepatology* 2010;**52**:291–302.
24. **Colmenero J,** Bataller R, Sancho-Bru P, *et al*. Hepatic expression of candidate genes in patients with alcoholic hepatitis: correlation with disease severity. *Gastroenterology* 2007;**132**:687–97.
25. **Dominguez M,** Rincon D, Abraldes JG, *et al*. A new scoring system for prognostic stratification of patients with alcoholic hepatitis. *Am J Gastroenterol* 2008;**103**:2747–56.
26. **Kleiner DE,** Brunt EM, Van Natta M, *et al*. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005;**41**:1313–21.
27. **Sancho-Bru P,** Altamirano J, Rodrigo-Torres D, *et al*. Liver progenitor cell markers correlate with liver damage and predict short-term mortality in patients with alcoholic hepatitis. *Hepatology*. Published Online First: 25 January 2012. doi:10.1002/hep.25614.
28. **Yoon SM,** Gerasimidou D, Kuwahara R, *et al*. Epithelial cell adhesion molecule (EpCAM) marks hepatocytes newly derived from stem/progenitor cells in humans. *Hepatology* 2011;**53**:964–73.
29. **Christensen E,** Gluud C. Glucocorticoids are ineffective in alcoholic hepatitis: a meta-analysis adjusting for confounding variables. *Gut* 1995;**37**:113–18.
30. **Imperiale TF,** Mccullough AJ. Do corticosteroids reduce mortality from alcoholic hepatitis? A meta-analysis of the randomized trials. *Ann Intern Med* 1990;**113**:299–307.
31. **Wiley SR,** Winkles JA. TWEAK, a member of the TNF superfamily, is a multifunctional cytokine that binds the TweakR/Fn14 receptor. *Cytokine Growth Factor Rev* 2003;**14**:241–9.

Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis

Silvia Affò, Marlene Dominguez, Juan José Lozano, et al.

Gut 2013 62: 452-460 originally published online May 25, 2012
doi: 10.1136/gutjnl-2011-301146

SUPPLEMENTARY MATERIALS AND METHODS

Microarray studies

High quality RNA samples were hybridized to GeneChips (Affymetrix *Hgu133plus* Affymetrix, Santa Clara, CA). Briefly, 2 µg of total RNA were used to generate double strand cDNA using an oligo dT- primer containing the T7 RNA polymerase promoter site and the SuperScript Choice System kit (Invitrogen, CA, USA). cDNA was purified by the GeneChip Sample Clean Up Module, followed by *in vitro* synthesis of biotinylated cRNA using the BioArray High Yield RNA transcription kit (Affymetrix). The resulting cRNA was purified and fragmented and 15 µg were hybridized to Human Genome U133A plus 2.0 GeneChip for 16 hours, at 45ºC and 30 g. The arrays were then washed and labelled with streptavidin-phycoerythrin (SAPE), and the signal was amplified with an anti-streptavidin biotinylated antibody followed by a second round of staining with SAPE using an Affymetrix fluidics station 450. Finally, the labelled arrays were scanned with a GeneChip scanner 3000 (Affymetrix). Microarray data were normalized using the guanidine-cytosine content-adjusted robust multiarray algorithm, which computes expression values from probe-intensity values incorporating probe-sequence information.[1] Next, we employed a conservative probe-filtering step excluding those probes not reaching a log2 expression value of 5 in at least one sample, which resulted in the selection of a total of 19,152 probes out of the original 54,675 set.

Functional analysis of microarray data

Differential expression was assessed by using linear models and empirical Bayes moderated t-statistics. Linear Models for Microarray Analysis (LIMMA) R-package software was used for the analysis of gene expression microarray data.[2] Two group comparisons and determinations of false discovery rates (FDR computation using Benjamini-Hochberg procedure) were performed and FDR values ≤ 0.05 were deemed potentially significant and selected for further study. Gene set enrichment analysis

program (GSEA) was employed to identify biological pathways significantly associated with the different phenotype in the study.[3] For the current analysis, gene sets were extracted from Molecular Signature Database (MSigDB v.2-0): C2-KEGG, C2-Biocarta, C2-Genmapp, C3 (gene sets containing genes that share transcription factor or microRNA binding motifs) and C5 (Gene Ontology terms) of MSigDB. Analysis was based on a t-test and a weighted scoring scheme with 1000 permutations on gene sets. Only gene sets with more than 15 genes were included in the analysis.

Supplementary Real-time polymerase chain reaction analysis

Quantitative real-time PCR (qPCR) was performed as previously described using commercial primer-probe pairs (Applied Biosystems, Foster City, CA) for TNFRSF12A (Fn14), CCL2 (MCP-1), IL-6, ICAM1, Col1a1, TGFβ, TNFα.[4] Data were normalized to 18s and gene expression values were calculated based on the $\Delta\Delta$Ct method. The results were expressed as $2^{-\Delta\Delta Ct}$.

Experimental mouse models

Male and female C57BL/6 mice 6-8 weeks aged, and male Balb/c mice 9 weeks aged (Charles River, l'Arbresle, France) were used for acute and chronic liver injury experiments. Male C57BL/6 mice (n=8) were intraperitoneally administered with carbon tetrachloride (CCl_4 Sigma-Aldrich; diluted 1:5 in oil) or vehicle (oil) at a dose of 1 ml/kg body weight and were sacrificed 72 hours later. Female C57BL/6 mice (n=8) received crotaline (Sigma-Aldrich) or vehicle intraperitoneal injections at a dose of 50 mg/kg every two weeks for a total of 2 injections. Two weeks after the last crotaline administration, mice were fasted for 8 hours with free access to water and then intraperitoneally injected with acetaminophen (Sigma-Aldrich) or vehicle at a dose of 500 mg/kg. Animals were sacrificed 48 hours after the last injection.[5]

Diet treatment: Male C57BL/6 mice (n=8) were fed a 0.1% 3,5-diethoxycarbonyl-1,4-dihydro-collidin (DDC) diet (Sigma-Aldrich) for up to 4 weeks.[6] Control mice (n=8) were fed a standard rodent chow (Harlan, Barcelona, Spain).

In all animal models, livers were excised and collected for RNA extraction and immunohistochemistry. All animal procedures were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

TWEAK serum levels

TWEAK serum levels were measured in patients with AH (n=22), and healthy volunteers (n=7) using the Human TWEAK ELISA development Kit from PeproTech (Pepro Tech Inc, Rocky Hill, CT).

REFERENCES

1. Wu Z, Irizarry RA, Gentleman R, et al. A Model-Based Background Adjustment for Oligonucleotide Expression Arrays. J Am Stat Assoc 2004;909-917.

2. Xia X, McClelland M, Wang Y. WebArray: an online platform for microarray data analysis. BMC Bioinformatics 2005;6:306.

3. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci USA 2005;102:15545-15550.

4. Sancho-Bru P, Altamirano J, Rodrigo-Torres D, et al. Liver progenitor cell markers correlate with liver damage and predict short-term mortality in patients with alcoholic hepatitis. Hepatology 2012:Jan 25. doi:10.1002/hep.25614.

5. Kofman AV, Morgan G, Kirshenbaum A, et al. Dose- and time-dependent oval cell reaction in acetaminophen-induced murine liver injury. Hepatology 2005;41:1252-1261.

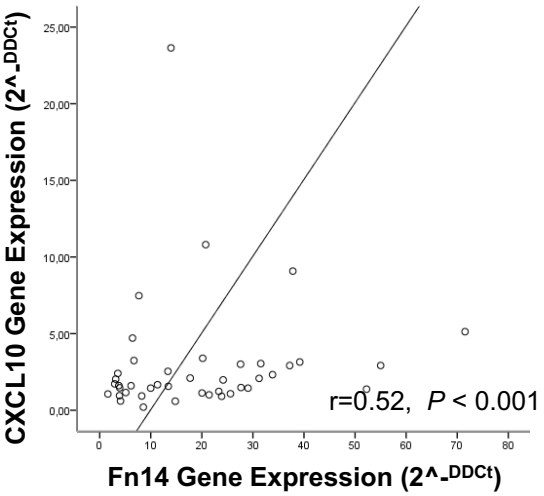
6. Jelnes P, Santoni-Rugiu E, Rasmussen M, et al. Remarkable heterogeneity displayed by oval cells in rat and mouse models of stem cell–mediated liver regeneration. Hepatology 2007;45:1462–1470.

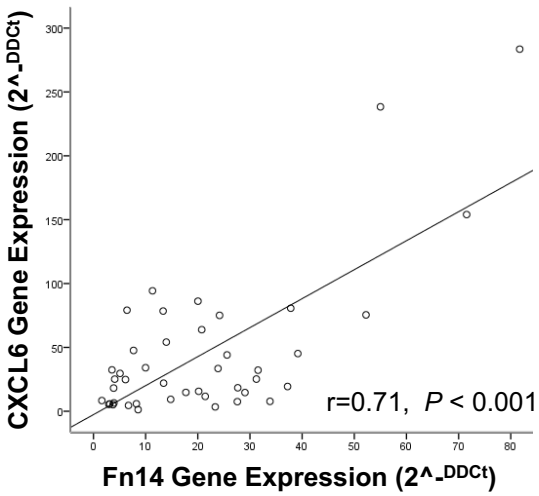
Supplementary Table 1. Clinical, virological and histological characteristics of patients with HCV and clinical, metabolic and histological characteristics of patients with NASH included in this study.

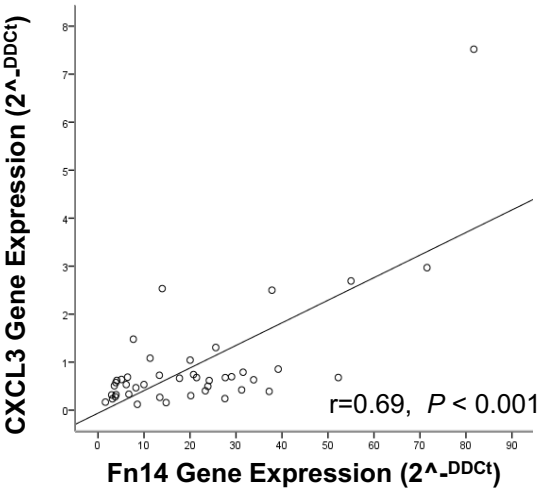
Variables	Mean ± SE or Percentage	Mean ± SE or Percentage
	HCV patients (n=18)	**NASH patients (n=20)**
Age (years)	52±2	47±2
Male (%)	61	35
BMI		50±2
Glucose (mg/dl)	90±2	131±11
HOMA	5±2.2	12±1.5
Cholesterol	176±6	199±7
Triglycerides	82±6	150±14
AST (U/L)	87±11	40±4
ALT (U/L)	110±13	58±7
GGT (U/L)	59±10	50±8
Bilirubin (mg/dl)	0.8±0.05	0.6±0.04
Viral Load (units/ml)	3329177±1172794	
METAVIR score F0 n,(%) F1 n,(%) F2 n,(%) F3 n,(%) F4 n,(%)	 2 (11) 2 (11) 7 (39) 1 (6) 6 (33)	 7 (35) 8 (40) 1 (5) 4 (20) 0 (0)
NAS score Steatosis n,(%) (0/1/2/3) Inflammation n, (%) (0/1/2/3) Hepatocyte damage n,(%) (0/1/2) Total score n, (%) (0-2/ 3-4/ 5-8)		 0 (0) /1 (5) / 8 (40) / 11 (55) 3 (15) / 6 (30) / 5 (25) / 6 (30) 3 (15) / 10 (50) / 7 (35) 0 (0) / 6 (30) / 14 (70)

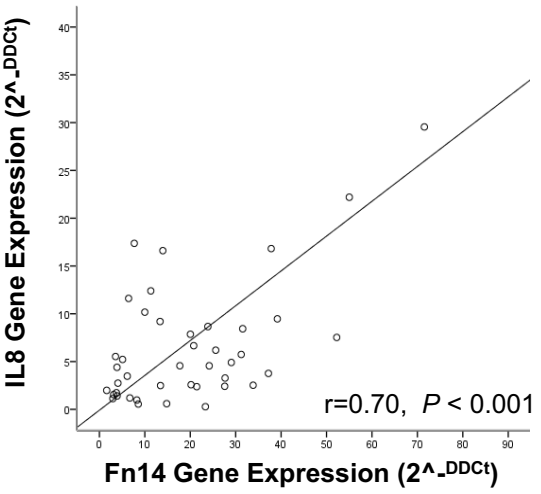
Supplementary Table 2. Clinical and analytical characteristics of healthy controls included in the microarray analysis.

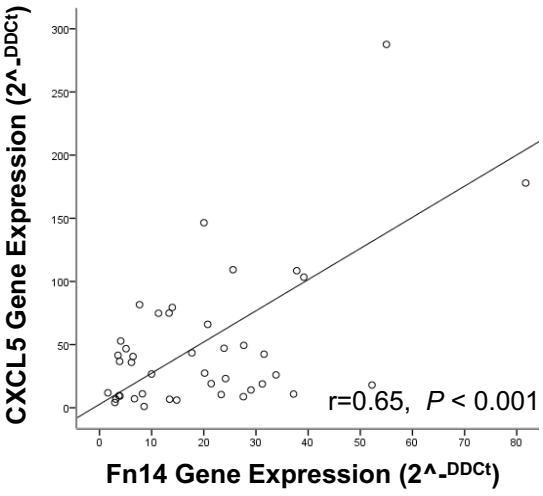
Clinical & Analytical Characteristics of health controls included in the array		
		Controls*
Gender (%)	Male	4 (57)
	Female	3 (43)
Age (years)		51 (43-68)
AST (U/L)		33 (27-63)
ALT (U/L)		35 (22-71)
Bilirubin (mg/dL)		0.6 (0.4-0.8)
γGT (U/L)		47 (13-130)
Albumin (mg/dl)		37 (32-42)
Leukocytes (x10^9)		6.9 (4.7-9.7)
Platelets (x10^9)		188 (170-219)
INR		1,1 (1.0-1.2)
Creatinine (mg/dl)		0.9 (0.7-1.7)
MELD		5 (2-10)

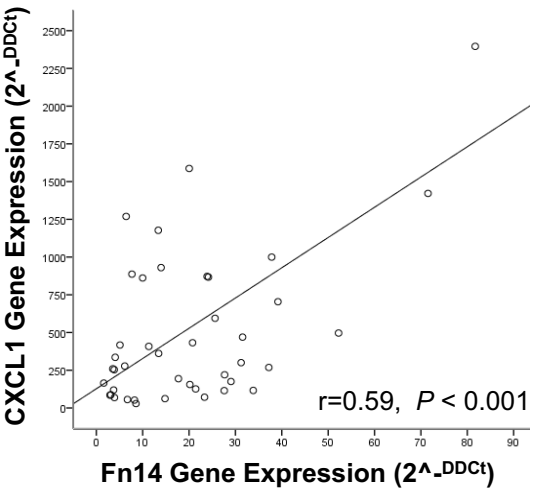




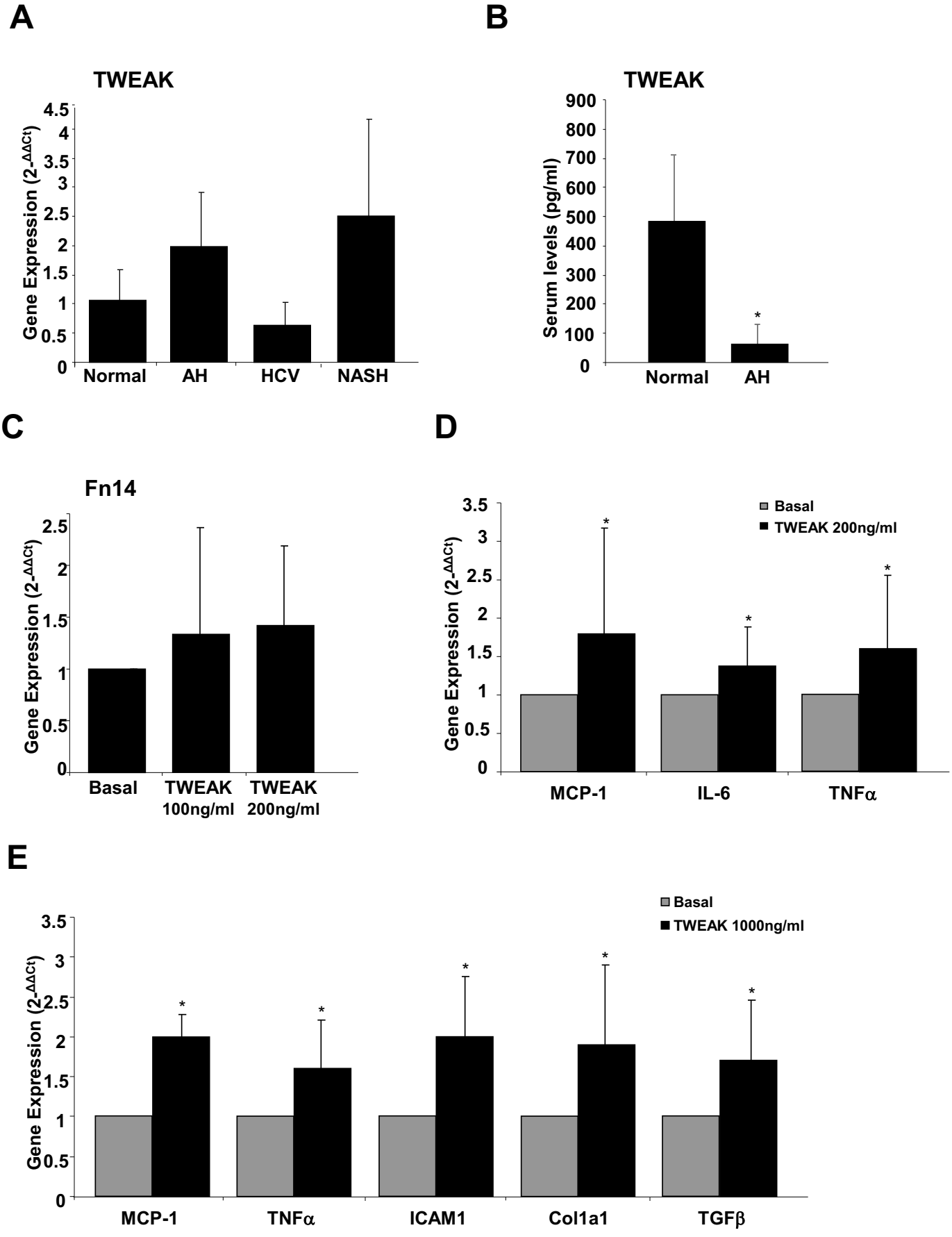








Supplementary Figure 1



Supplementary Figure 2

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure 1. Correlation between hepatic gene expression of Fn14 and CXC chemokines in patients with AH.

Supplementary Figure 2. (A) TWEAK hepatic gene expression in normal livers and patients with AH, HCV and NASH; (B) TWEAK serum levels in patients with AH respect to normal controls (*p=0.001). (C) Effects of TWEAK stimulation on Fn14 gene expression in mice high precision-cut liver slices. (D,E) Effects of TWEAK (200 ng/ml and 1000 ng/ml) stimulation on pro-inflammatory and pro-fibrogenic gene expression (*$p<0.05$).

SECOND ARTICLE

"CCL20 mediates LPS-induced liver injury and is a potential driver of inflammation and fibrosis in alcoholic hepatitis"

Affò S. et al. Gut - Manuscript ID gutjnl-2013-306098.R1-Accept (12-Dec-2013)

Gut doi:10.1136/gutjnl-2013-306098

Chemokines are known to play an important role in the pathophysiology of AH, a form of acute-on-chronic liver injury frequently mediated by gut-derived LPS translocation to the liver; inflammatory cell recruitment (mostly neutrophils), hepatic fibrosis and hepatocellular damage. In our study we hypothesized that chemokine CCL20, one of the most up-regulated chemokines in patients with AH, is implicated in the pathogenesis of AH by mediating LPS-induced liver injury. We assessed CCL20 gene expression and serum levels and their correlations with disease severity in a cohort of patients with AH. Cellular sources of CCL20 and its biological effects were evaluated *in vitro* and *in vivo* in chronic, acute and acute-on-chronic experimental models of CCl_4 and LPS-induced liver injury. RNA interference technology was used to knockdown CCL20 *in vivo*. The results obtained in this study suggest that CCL20 up-regulation is strongly associated with LPS and endotoxemia and may not only represent a new potential biomarker to predict outcome in patients with AH, but also an important mediator linking hepatic inflammation, injury and fibrosis in AH.

Patients with AH show increased CCL20 hepatic expression and serum levels

In the previous study "*Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis*" we identified CCL20 as the most up-regulated CC chemokine in patients with AH, with more than 90-fold up-regulation compared with controls. To confirm this previous result, we analyzed by quantitative PCR the hepatic *CCL20* expression in a cohort of patients with clinical, analytical and histological characteristics of AH. The results confirmed a marked up-regulation of *CCL20* in patients with AH (n=32) as compared with normal liver (n=8) ($p<0.001$) and other liver diseases. *CCL20* expression was also found up-regulated, but at a significant lower extent compared to AH patients, in patients with NASH (n=8) ($p<0.005$), chronic hepatitis C (n=8) ($p<0.005$) and compensated cirrhosis

(n=8) ($p<0.001$) as compared with control liver samples (n=8). We also measured CCL20 serum levels in patients with AH and other liver diseases. We found that CCL20 serum levels were increased in patients with AH (n=49) ($p<0.001$), HCV (n=8) and compensated alcoholic cirrhosis (n=15) ($p<0.005$) compared to healthy controls (n=8). Of note, CCL20 circulating levels were higher in patients with AH compared to patients with other liver diseases ($p<0.001$). Interestingly, we observed that hepatic CCL20 mRNA expression and serum levels positively correlated in patients with AH (n=32) ($p=0.03$), suggesting that the liver may be an important source of CCL20 in these patients.

CCL20 expression correlates with disease severity and key features of AH

To better understand the role of CCL20 in the pathogenesis of AH, we next explored whether its expression correlated with disease severity and with clinical features of the disease. We found that *CCL20* hepatic expression positively correlated with important prognostic scores in patients with AH. Indeed, hepatic *CCL20* correlated with MELD ($p<0.0001$), ABIC ($p=0.06$) and Maddrey's ($p=0.005$) scores. Moreover, we observed higher levels of hepatic *CCL20* expression in patients with severe AH compared with those with mild-to-moderate grade of fibrosis and portal hypertension (54 vs. 7 fold expression; $p=0.01$ and 148 vs. 34 fold expression; $p=0.008$ respectively). We next wanted to investigate the correlation between circulating CCL20 and LPS, one of the major inducers of CCL20 and known to be implicated in the pathogenesis of AH. Interestingly, we observed that CCL20 and LPS serum levels were strongly correlated ($p<0.0001$) in patients with AH. We also evaluated in our cohort of patients the hepatic infiltration of neutrophils, another important hallmark in AH. We found that patients with higher levels of circulating CCL20, showed severe hepatic infiltration of PMN compared to those with mild grade of PMN infiltration ($p=0.007$).

Importantly, increased hepatic CCL20 mRNA and serum levels were observed in patients who died within 90 days after admission compared with those who survived (160- vs 50-fold induction; $p=0.03$ and 359 vs. 168pg/ml; $p=0.048$ respectively). In addition, to determine if CCL20 could be a good predictor of short-term mortality, a Kaplan Meier analysis was performed. CCL20 hepatic gene expression (receiver operating curve (ROC) cut off value of 80-fold ($2^{-\Delta\Delta Ct}$), AUROC: 0.72, 95% CI [0.53-0.90]) and serum levels (ROC cut off value of 260pg/ml, AUROC: 0.68, 95% CI [0.52-0.83]) were found useful to predict short-term mortality

in patients with AH. These results suggest that CCL20 may play a role in the pathophysiology of AH and could be used as a biomarker to predict short-term mortality.

CCL20 exerts pro-inflammatory and pro-fibrogenic effects on hepatic stellate cells

Since hepatic expression of CCL20 correlated with the grade of fibrosis, we next investigated the potential of HSCs to synthesize CCL20 and CCL20 biological effects on these cells. We first investigated if mediators known to play a role in ALD and typically present in AH microenvironment such as LPS, TNF-α and IL-1β, induced *CCL20* expression in human primary HSCs. We observed that the incubation of HSCs with LPS, TNF-α and IL-1β induced a marked increase in *CCL20* mRNA levels ($p<0.05$). On the other hand, to investigate the biological effects of CCL20 on HSCs, cells were incubated with recombinant CCL20. The chemokine induced the expression of pro-inflammatory (*MCP-1*, *RANTES*, *ICAM-1*) ($p<0.05$) and pro-fibrogenic (*COL1A1* and *TGF-β*) ($p<0.05$) genes in HSCs.

To investigate if CCL20 had a chemo-attractant effect on HSCs, we performed a migration test using a Boyden Chamber. We found an increased HSCs migration after cell stimulation with CCL20 ($p<0.005$). Previous studies showed the implication of ERK signaling pathway in HSCs migration and activation. For that reason, we tested if CCL20-induced HSCs migration occurred in an ERK-dependent manner. Interestingly, we found that CCL20 induced a transient activation of ERK phosphorylation and we observed that the pre-incubation of HSCs with U0126, a MEK 1/2 specific inhibitor, reduced CCL20-induced migration of HSCs ($p=0.014$). These results indicate that CCL20 exerts pro-inflammatory and pro-fibrogenic effects on HSCs and enhances their migration through ERK signaling.

LPS induces hepatic up-regulation of CCL20

The existing animal models of alcoholic liver injury do not reproduce all the features of severe AH observed in humans. For this reason and in order to uncover the mechanisms driving the increase of *CCL20* expression in AH and its cellular source, we performed different animal models of liver injury combining some of the key events that occur in AH such as ethanol consumption, fibrosis and endotoxemia. We first tested the effect of ethanol on *Ccl20* hepatic expression. Mice administered with ethanol by gavage did not show increased *Ccl20*

hepatic levels while other molecules important in AH such as Fn14 were found increased in this model. We next investigated if CCl_4 or LPS administration induced *Ccl20* hepatic expression. Interestingly, we found that both CCl_4 and LPS significantly increased *Ccl20* hepatic gene expression ($p<0.05$) even though CCl_4 to a lesser extent compared to LPS. Importantly, mice treated with a combination of CCl_4 and LPS resulted in a strong increase of *Ccl20* hepatic expression, as compared to mice treated with LPS, CCl_4 and respect to control mice ($p<0.05$). To confirm the extent of liver damage in mice injected with the combination of CCl_4 and LPS, we performed multiple approaches. By Sirius red we appreciated an increased collagen deposition, by PCR analysis we observed enhanced hepatic gene expression of *Col1a1*, *Tgf-ß*, *Icam-1*, and *F4/80* and by immunohistochemistry and Western blot we confirmed enhanced protein expression of F4/80, CCL20 and ICAM-1 in animals administered with CCl_4 plus LPS compared to control mice.

Macrophages are the main cell source of hepatic CCL20 in LPS-induced liver injury

Once identified the experimental models inducing CCL20 expression, we aimed to identify the main cell source of CCL20 in the injured liver. After induction of acute-on-chronic liver injury (CCl_4 plus LPS) in mice, we isolated different hepatic cell populations from livers of treated mice using Nycodenz gradients and FACS sorting and we evaluated the hepatic expression of *Ccl20*. We identified macrophages as the hepatic cell type expressing higher levels of *Ccl20* ($p<0.001$), followed by activated HSCs, T cells and hepatocytes ($p<0.05$ for all as compared to whole liver). Interestingly, HSCs obtained by FACS sorting and sorted for Vitamin A, produced very low levels of CCL20 as compared with total HSCs, isolated by Nycodenz gradient, indicating that HSCs activation and subsequent loss of Vitamin A may be a crucial event in CCL20 production. Since macrophages were identified as the main hepatic *Ccl20* cell source, and their activation is a crucial step in liver inflammation and fibrosis, we also explored *Ccl20* production *in vitro* in RAW264 cell line. We found that LPS induced a strong increase in *Ccl20* gene expression and in a dose-dependent manner in these cells type.

Silencing CCL20 ameliorates LPS-induced liver injury

Once identified LPS as one of the major inducers of *Ccl20*, we evaluated the effects mediated by CCL20 in animal models of LPS-induced liver injury. We found that mice treated

with LPS presented an important hepatocellular damage showing increased ALT, AST and LDH levels, which were markedly reduced in animals pre-treated with shRNA specific for CCL20 as compared with control shRNA. Moreover, we observed that LPS induced an important increase in both gene and protein expression of *Ccl20, Nos2, Icam-1, Mcp-1, Tgf-β and Col1a1*. Animals treated with CCL20 shRNA showed a marked reduction in *Ccl20* expression at mRNA and protein level, indicating an efficient knockdown due to the shRNA treatment. Moreover, we observed a clear decrease of *Nos2, Icam-1, Mcp-1* and *Tgf-β* gene expression ($p<0.05$) in animals treated with CCL20 shRNA and LPS. We also found a reduction of hepatic protein expression of NOS2 and ICAM1 ($p<0.05$) in mice injected with CCL20 shRNA and LPS as compared to the control group.

Furthermore, we investigated the effects of CCL20 on hepatic inflammatory cell recruitment and we found that CCL20 knockdown reduced macrophages and neutrophils hepatic infiltration in LPS-induced injury ($p<0.05$). CCL20 knockdown also induced a reduction in caspase-8 ($p=0.059$) and caspase-3 ($p=0.077$) LPS-induced cleavage. These results suggest that CCL20 mediates LPS-induced hepatocellular damage, regulates important molecules that participate in the pathogenesis of AH and modulates the hepatic inflammatory infiltrate recruitment.

Decision Letter (gutjnl-2013-306098.R1)

From: info.gut@bmj.com
To: psancho@clinic.ub.es
CC:
Subject: Gut - Decision on Manuscript ID gutjnl-2013-306098.R1
Body: 12-Dec-2013

Dear Dr. Sancho-Bru,

Manuscript ID gutjnl-2013-306098.R1 - CCL20 mediates LPS-induced liver injury and is a potential driver of inflammation and fibrosis in alcoholic hepatitis

Thank you for submitting your article to Gut, which I have pleasure in accepting for publication as a Original Article. Well done and many congratulations on an excellent paper!

Thank you for choosing Gut.

With kind regards.
Yours sincerely,

Dr. Matias Avila
Associate Editor, Gut

Prof. Emad El-Omar
Editor in Chief, Gut

On behalf of the Gut Editorial Committee

http://gut.bmj.com
Twitter: @GUT_BMJ

To be alerted when your article is published in the print journal go to:
http://gut.bmjjournals.com/cgi/etoc.

Date Sent: 12-Dec-2013

CCL20 mediates LPS-induced liver injury and is a potential driver of inflammation and fibrosis in alcoholic hepatitis

Silvia Affò[1], Oriol Morales-Ibanez[1], Daniel Rodrigo-Torres[1], José Altamirano[1], Delia Blaya[1], Dianne H. Dapito[2], Cristina Millán[1], Mar Coll[1], Jorge M. Caviglia[2], Vicente Arroyo[1], Juan Caballería[1], Robert F. Schwabe[2], Pere Ginès[1], Ramón Bataller[1,3] and Pau Sancho-Bru[1].

[1] Liver Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Faculty of Medicine University of Barcelona, Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona, Spain.

[2] Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY, USA.

[3] Division of Gastroenterology and Hepatology, Departments of Medicine and Nutrition, University of North Carolina at Chapel Hill, NC, USA.

Correspondence to:

Pau Sancho-Bru, PhD
Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)
Centre Esther Koplowitz
C/ Roselló, 149-153, third floor,
08036 Barcelona, Spain
psancho@clinic.ub.es
Tel. +34 932275400 Ext. 3371

Keywords: Alcoholic liver disease, chemokines, endotoxemia, acute-on-chronic liver damage, RNA small hairpin interference-inducing constructs (shRNA).

Abbreviations: ABIC, Age-Bilirubin-INR-Creatinine score; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AH, alcoholic hepatitis; AST, aspartate aminotransferase; AUROC, area under the receiver characteristic curve; CCl_4, carbon tetrachloride; CCR6, CC chemokine receptor 6; GGT, gamma-glutamyl transpeptidase; HCV, hepatitis C virus; HSC, hepatic stellate cell; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MELD, Model for End-stage Liver Disease; MPO, myeloperoxidase; NASH, nonalcoholic steatohepatitis; shRNA, short hairpin interference inducing construct; PMN, polymorphonuclear cells*;* siRNA, small interfering RNA; TLRs, toll-like receptors.

Word count

Abstract: **245**

Text: Excluding title page, Abstract, References, Figure legends, Figures and Tables, Significance of the study, acknowledgments and funding: **4102**

ABSTRACT

Objective Chemokines are known to play an important role in the pathophysiology of alcoholic hepatitis (AH), a form of acute-on-chronic liver injury frequently mediated by gut-derived lipopolysaccharide (LPS). In our study we hypothesize that chemokine CCL20, one of the most up-regulated chemokines in patients with AH, is implicated in the pathogenesis of AH by mediating LPS-induced liver injury.

Design CCL20 gene expression and serum levels and their correlations with disease severity were assessed in patients with AH. Cellular sources of CCL20 and its biological effects were evaluated *in vitro* and *in vivo* in chronic, acute and acute-on-chronic experimental models of carbon tetrachloride (CCl_4) and lipopolysaccharide (LPS)-induced liver injury. RNA interference technology was used to knockdown CCL20 *in vivo*.

Results CCL20 hepatic and serum levels were increased in patients with AH and correlated with the degree of fibrosis, portal hypertension, endotoxemia, disease severity scores and short-term mortality. Moreover, CCL20 expression was increased in animal models of liver injury and particularly under acute-on-chronic conditions. Macrophages and hepatic stellate cells (HSCs) were identified as the main CCL20 producing cell types. Silencing CCL20 *in vivo* reduced LPS-induced AST and LDH serum levels and hepatic pro-inflammatory and pro-fibrogenic genes. CCL20 induced pro-inflammatory and pro-fibrogenic effects in cultured primary HSCs.

Conclusion Our results suggest that CCL20 up-regulation is strongly associated with LPS and may not only represent a new potential biomarker to predict outcome in patients with AH, but also an important mediator linking hepatic inflammation, injury and fibrosis in AH.

SIGNIFICANCE OF THIS STUDY

What is already known about the subject?

- Alcoholic hepatitis (AH) is the most severe form of alcoholic liver disease (ALD) and is associated with high rate of short-term mortality. Current therapies such as corticosteroids are not fully effective and new-targeted therapies for the treatment of this disease are urgently needed.
- Alcohol consumption leads to an increase of endotoxin levels in the blood. Once it reaches the liver, endotoxin mostly activates Kupffer cells and hepatic stellate cells and determines the promotion and perpetuation of hepatic inflammation and fibrosis.
- CCL20 is a pro-inflammatory chemokine strongly induced in different cell types by lipopolysaccharide (LPS), TNFα and IL1β and is known to recruit CC chemokine receptor 6 (CCR6) positive cells.

What are the new findings?

- CCL20 hepatic expression and serum levels are elevated in patients with AH and are associated with key clinical features of the disease such as grade of fibrosis, portal hypertension severity, endotoxemia and hepatic neutrophils infiltration. Increased CCL20 hepatic gene expression and serum levels are associated with short-term mortality in patients with AH.
- Macrophages and hepatic stellate cells are the main CCL20 producing cell types in experimental acute-on-chronic liver damage induced by the combined treatment of chronic carbon tetrachloride (CCl_4) and LPS.
- CCL20 exerts pro-inflammatory and pro-fibrogenic effects on primary human hepatic stellate cells *in vitro*.
- CCL20 knockdown reduces LPS-induced liver damage and determines an important decrease of pro-inflammatory and pro-fibrogenic genes.

How might it impact on clinical practice in the foreseeable future?

The identification of molecular drivers of AH will provide new potential targets for therapy for this severe disease. In our study we provide relevant results, which show a correlation between CCL20 hepatic and serum levels with the grade of fibrosis, portal hypertension, endotoxemia, neutrophils infiltration and mortality in patients with AH. These findings represent new interesting discoveries in the pathophysiology of alcoholic liver diseases and suggest that CCL20 may play an important role in the pathogenesis of AH. Moreover, the correlation of CCL20 with patient's outcome suggests that CCL20 serum level could be used as a biomarker to predict short-term mortality in patients with AH.

INTRODUCTION

Alcoholic liver disease (ALD) is a major cause of end-stage liver disease worldwide and includes a broad spectrum of disorders, from fatty liver and hepatic inflammation to more severe forms of liver injury, including alcoholic hepatitis (AH), cirrhosis, and hepatocellular carcinoma.[1] AH is the most severe form of ALD and leads to severe complications related to liver failure, portal hypertension or bacterial infection and is associated with high short-term mortality.[1, 2, 3, 4] AH episodes are associated with an important inflammatory response and a rapid progression of liver fibrosis.[5] Unfortunately, corticosteroid treatment is only effective for a subset of patients,[6] and no other efficient therapies are currently available. The development of new therapeutic strategies in AH have been hampered by the poor knowledge of the molecular mechanisms [1, 5, 7] and the lack of animal models of severe AH, since available models do not reproduce all the key histological features found in humans.[5, 8] However, new animal models reproducing some of the features of AH in humans, have been recently described [9, 10] and will represent new important tools to study the disease.

Alcohol consumption induces the disruption of the intestinal barrier and causes enhanced gut permeability with subsequent translocation of bacterial-derived lipopolysaccharide (LPS), which leads to elevated serum levels of LPS in patients with AH.[11, 12, 13] Once it reaches the liver, LPS stimulates innate immune receptors, namely toll-like receptors (TLRs), mostly expressed on Kupffer cells and hepatic stellate cells (HSCs).[14] LPS-mediated activation of Kupffer cells is a crucial step for both liver inflammation and fibrogenesis by promoting hepatocyte damage, increased leukocyte infiltration, secretion of reactive oxygen species (ROS) and pro-inflammatory and pro-fibrogenic cytokines.[15, 16] Furthermore, LPS can also directly contribute to HSCs activation and promote liver fibrosis.[15, 17] A previous translational study from our laboratory using liver samples from patients with AH, allowed us to identify several deregulated pathways potentially implicated in the pathogenesis of AH, including cytokine–cytokine receptor interaction pathway.[8, 18] In the same study we identified CCL20 as the most up-regulated chemokine in patients with AH.

Chemokines are a family of small cytokines, which have the properties of both chemotactic mediators and cytokines.[19] Chemokines mediate the infiltration of immune cells into the injured liver, but can also directly interact with hepatic resident cells during inflammation and fibrosis.[20] CCL20 was originally identified in the liver as liver-and activation-related chemokine (LARC) and is also known as macrophage inflammatory protein (MIP-3alpha).[21] CCL20 has been described to be the only chemokine interacting and activating CC chemokine receptor 6 (CCR6), receptor shared only with the antimicrobial β-defensins.[22] CCL20 has been shown to be expressed in a broad spectrum of cells and tissue types. Based on the variety of CCL20-inducing agents (LPS, TNFα, IL1β), CCL20 and CCR6 have been described to be involved in both normal and pathological processes [22] including chronic liver injury [23, 24] and hepatocellular carcinoma.[25] However, the role of CCL20 in chronic liver diseases and in the context of an acute-on-chronic liver injury is unknown.

In the present translational study, we investigated the potential role of CCL20 as a mediator of LPS-induced liver injury in AH. We performed an extensive study in liver samples from well-characterized patients with AH and we demonstrated that CCL20 is up-regulated in these patients and correlates with grade of fibrosis, portal hypertension, endotoxemia, disease severity and mortality. Moreover, since there are not available experimental models of AH, we explored the CCL20 cell sources and functions in experimental models of acute, chronic and acute-on-chronic liver injury induced by LPS, CCl_4 and their combination to reproduce some of the features of AH.

MATERIALS AND METHODS

Patients

Patients admitted to the Liver Unit, Hospital Clínic of Barcelona with clinical, analytical and histological features of AH from July 2009 to January 2012 were prospectively included in the study. All patients included in this study gave informed consent and the protocol was conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Clinic of Barcelona. CCL20 and LPS serum levels were assessed in 49 patients and hepatic gene expression analysis was performed in 32 liver samples obtained by transjugular biopsy. The inclusion criteria of AH were: excessive alcohol consumption (>60 g/day) prior to admission, elevated aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT) and bilirubin, and histological diagnosis of AH.[2, 26] Patients with hepatocellular carcinoma or any other potential cause of liver disease were excluded from the study. All patients received nutritional as well as psychological support for achieving alcohol abstinence. Fragments of normal livers were selected as previously described.[8] We included patients with hepatitis C virus (HCV)-induced liver disease (genotype 1) who did not use any previous antiviral therapy, patients with compensated cirrhosis due to HCV or past-history of alcohol abuse (abstinence for at least 6 months) and a cohort of patients with morbid obesity and associated nonalcoholic steatohepatitis (NASH) according to Kleiner's criteria. Clinical and histological characteristics of these patients have been previously described.[7,8]

Determination of LPS and CCL20 serum levels in patients with AH

Serum samples were obtained from peripheral blood and stored at -80ºC. LPS serum levels were determined using Limulus Amebocyte Lysate (LAL) QCL-1000 test (Lonza Walkersville Inc., Walkersville, MD, USA). CCL20 serum levels were measured in patients with AH (n=49), HCV (n=8), and compensated alcoholic cirrhosis (n=15) and in healthy volunteers (n=8) using the Quantikine Human CCL20/MIP-3α Immunoassay Kit (R&D Systems, Minneapolis, MN, USA).

Cell cultures and *in vitro* assays

Human HSCs were isolated and cultured as previously described.[7] To study CCL20 production and biological effects, HSCs were serum-starved for 12 hours and then incubated with LPS 1μg/ml (Sigma-Aldrich, St. Louis, MO, USA), TNFα 1ng/ml (R&D Systems), and IL1β 20ng/ml (Sigma-Aldrich) for 24 hours and with CCL20 250ng/ml and 1μg/ml (R&D Systems) for 24 and 48 hours respectively. HSCs migration assays were performed using a Boyden Chamber and CCL20-induced ERK activation was verified by Western blotting (*Supplementary Material*). RAW264 murine macrophages were incubated with LPS (10ng/ml, 100ng/ml and 1μg/ml) for 24 hours as previously described.[7] RNA isolation and PCR analysis were performed as described in the *Supplementary Material* section.

Small hairpin interference-inducing constructs

We first tested in RAW264 cells three small interfering RNA (siRNAs) specific for both isophorm 1 and 2 of CCL20 (s73425, s73427 and s73426, Ambion® In Vivo siRNA, Ambion, Life Technologies Corporation, Carlsbad, CA, USA) (data not shown) and using a positive (Ambion® In Vivo GAPDH Positive control siRNA, Ambion) and negative control (Ambion® In Vivo Negative Control #1 siRNA, Ambion). We chose the siRNA that best inhibited *Ccl20* gene expression for the production of CCL20 short hairpin interference-inducing construct (shRNA). Starting from siRNA sequence, shRNA for *in vivo* use were constructed and provided by the Gene Silencing Platform at CIC bioGUNE (Bilbao, Spain). Briefly, chemically synthesized oligonucleotides including the gene target sequence (or a scrambled sequence in case of the control shRNA) and a 19nt loop from human miR30 were cloned into the pSM2C vector.

Mouse models of liver injury

Animal procedures were approved by the Ethics Committee of the University of Barcelona and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and by the Columbia University Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Mice 8-10 weeks aged were administrated carbon tetrachloride (CCl_4) and ethanol or LPS. To mimic the effects of endotoxemia in the context of chronic liver disease we also used a model of acute-on-chronic liver injury by combining the effects of chronic CCl_4 plus LPS. Different hepatic cell populations were isolated from livers of mice treated with CCl_4 and LPS, and *Ccl20* hepatic expression was evaluated. The effects of CCL20 were studied *in vivo* by injecting mice with control shRNA or shRNA specific for CCL20 and LPS. The effects of the shRNA on hepatic inflammatory cell infiltration, gene and protein expression were assessed by quantitative PCR, immunohistochemistry and Western blotting respectively. For details on methodology, please see *Supplementary Material*.

Statistical analysis

Continuous variables were described as mean (95% confidence interval) or median (interquartile range). Categorical variables were described by means of counts and percentages. Comparisons between groups were performed using the Student´s *t* test or the Mann-Whitney *U* test when appropriate. Correlations between variables were evaluated using Spearman's *rho* or Pearson's *r*, when appropriate. The area under the receiver characteristic curve (AUROC) analysis was used to determine the best cut-off value and the accuracy (sensitivity and specificity) of continuous variables associated with 90-day mortality. Finally, we performed a survival analysis using the Kaplan-Meier method. Comparisons were performed by the log-rank test. All statistical analyses were performed using SPSS version 14.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

General characteristics of patients with AH

Forty-nine patients were included in the study with clinical, analytical and histological characteristics of AH. Seventy-eight percent (n=38) of patients had severe AH at admission, as defined as ABIC (Age-Bilirubin-INR-Creatinine) score >6.71.[2] Patients were predominantly male (80%) and the mean age was 52 years. Overall 90-day mortality was 29%. The main causes of death were multiple organ dysfunction (65%) and severe sepsis (20%). The main epidemiological, clinical, hemodynamic and analytical characteristics of patients are shown in Table 1.

Table 1. Baseline Demographic and Clinical Parameters of Patients with Alcoholic Hepatitis (n=49)

Characteristics		**Median (25-75 IQR)**
Age (y)		52 (47-56)
Male n (%)		39 (80)
Alcohol intake (g/day)		100 (80-160)
Corticosteroids n (%)		25 (51)
Laboratory and hemodynamic parameters		
Hemoglobin (g/dL)		11 (10-13)
Leukocyte count $x10^9$/L		8.4 (6.3-12.5)
Platelet count $x10^9$/L		113 (77-201)
AST (U/L)		117 (67-157)
ALT (U/L)		37 (25-60)
Serum Na (mmol/L)		135 (132-139)
Serum albumin (g/dL)		2.6 (2.3-3.2)
Serum creatinine (mg/dL)		0.9 (0.60-1.1)
Serum bilirubin (mg/dL)		6.7 (3.0-18.7)
International normalized ratio		1.6 (1.4-1.8)
HVPG (mmHg)		19 (15-22)
Alcoholic hepatitis severity scores at admission		
MELD score		19 (14-24)
ABIC score		7.8 (6.7-8.6)
ABIC class n(%)	A (<6.71)	11 (23)
	B (6.71-8.99)	30 (61)
	C (≥9)	8 (16)
Clinical decompensations during hospitalization		
AKI n (%)		20 (41)
Infection n (%)		21 (43)
Mortality at 90 days		**14 (29)**

AST, aspartate aminotransferase; ALT, alanine aminotransferase; HVPG, hepatic venous pressure gradient; MELD, Model for End-stage Liver Disease; ABIC, Age-Bilirubin-INR-Creatinine score; AKI, acute kidney injury.

Patients with AH show increased CCL20 hepatic expression and serum levels

We previously identified CCL20 as the most up-regulated CC chemokine in patients with AH.[8] To confirm this previous result, we analyzed by real-time PCR the hepatic *CCL20* expression in a cohort of patients with AH. The results confirmed a marked up-regulation of *CCL20* in patients with AH (n=32) as compared with normal liver (n=8) ($p<0.001$) and other liver diseases ($p<0.001$). *CCL20* expression was also up-regulated but at a lower extent in patients with NASH (n=8) ($p<0.005$), chronic hepatitis C (n=8) ($p<0.005$) and compensated cirrhosis (n=8) ($p<0.001$) as compared with control liver samples (n=8) (Figure 1A).

We next assessed CCL20 serum levels in patients with AH and other liver diseases. We found that CCL20 serum levels were increased in patients with AH (n=49) ($p<0.001$), HCV (n=8) and compensated alcoholic cirrhosis (n=15) ($p<0.005$) compared to healthy controls (n=8). Of note, CCL20 circulating levels were higher in patients with AH compared to patients with other liver diseases ($p<0.001$, Figure 1B). Finally, we observed that hepatic CCL20 mRNA expression and serum levels positively correlated in patients with AH (n=32) ($p=0.03$, Figure 1C), suggesting that the liver may be an important source of CCL20 in these patients.

CCL20 expression correlates with disease severity and key features of AH

To gain insight in the pathogenic role of CCL20 in AH, we next explored whether its expression correlated with disease severity. *CCL20* hepatic expression positively correlated with important prognostic scores in patients with AH. Hepatic *CCL20* correlated with MELD (Model for End-stage Liver Disease) ($p<0.0001$) (Figure 2A), ABIC ($p=0.06$) and Maddrey's ($p=0.005$) (Supplementary Figure 1A,B) scores. Moreover, we observed higher levels of hepatic *CCL20* expression in patients with severe AH compared to those with mild-to-moderate grade of fibrosis and portal hypertension (54 vs. 7 fold expression; $p=0.01$ and 148 vs. 34 fold expression; $p=0.008$ respectively; Figure 2B,C). We next sought to investigate the correlation between circulating CCL20 and LPS, one of the major inducers of CCL20. We observed that CCL20 and LPS serum levels were strongly correlated ($p<0.0001$, Figure 2D) in patients with AH. We also evaluated in our cohort of patients the hepatic infiltration of neutrophils (as described in *Supplementary Material* section) an important

hallmark in AH. We found that patients with higher levels of circulating CCL20 showed severe hepatic infiltration of polymorphonuclear cells (PMN) compared to those with mild grade of PMN infiltration (p=0.007, Supplementary Figure 1C).

Importantly, we observed increased hepatic CCL20 mRNA and serum levels in patients who died within 90 days after admission compared with those who survived (160- vs 50-fold induction; p=0.03 and 359 vs. 168pg/ml; p=0.048 respectively; Supplementary Figure 1D, E). In addition, to determine if CCL20 could be a good predictor of short-term mortality, a Kaplan Meier analysis was performed. As shown in Figure 2 (E,F) CCL20 hepatic gene expression (receiver operating curve (ROC) cut off value of 80-fold ($2^{-\Delta\Delta Ct}$), AUROC: 0.72, 95% CI [0.53-0.90]) and serum levels (ROC cut off value of 260pg/ml, AUROC:0.68, 95% CI [0.52-0.83]) were useful to predict short-term mortality in patients with AH. These results suggest that CCL20 may play a role in the pathophysiology of AH and could be used as a biomarker to predict short-term mortality.

CCL20 pro-inflammatory and pro-fibrogenic effects on hepatic stellate cells

Since hepatic expression of CCL20 was found to be increased in AH patients with METAVIR F4 compared to those with METAVIR F1-F3 (Figure 2B), and since HSCs are key players in the development of liver fibrosis in the injured liver, we next investigated the potential of HSCs to synthesize CCL20 and its biological effects on these cells. We first investigated if mediators known to play a role in ALD and typically present in AH microenvironment, induced *CCL20* expression in human primary HSCs. Incubation of HSCs with LPS, TNFα and IL1β induced a marked increase in *CCL20* mRNA levels ($p<0.05$) as shown in Figure 3A. On the other hand, to investigate the biological effects of CCL20 on HSCs, cells were incubated with recombinant CCL20. The chemokine induced the expression of pro-inflammatory (*MCP1*, *RANTES*, *ICAM1*) ($p<0.05$) and pro-fibrogenic (*COL1A1* and *TGFβ*) ($p<0.05$) genes in HSCs (Figure 3B). To investigate if CCL20 had a chemoatractant effect on HSCs, we performed a migration test using a Boyden Chamber. We found an increased HSCs migration after cell stimulation with CCL20 ($p<0.005$) (Figure 3C). Previous studies showed the implication of ERK in HSCs migration and activation [27] so we tested if CCL20-induced HSCs migration occurred in an ERK-dependent manner. Interestingly, CCL20

induced a transient activation of ERK phosphorylation (Figure 3D) and pre-incubation of HSCs with U0126, a MEK 1/2 specific inhibitor, reduced CCL20-induced migration of HSCs (p=0.014) (Figure 3E,C). These results indicate that CCL20 exerts pro-inflammatory and pro-fibrogenic effects on HSCs and enhances their migration through ERK signaling.

LPS induces hepatic up-regulation of CCL20

Our group and others have been working on the development of an animal model of severe AH but unfortunately; the existent models do not reproduce the pathophysiology of severe AH observed in humans. For this reason and in order to uncover the mechanisms driving the increase in *CCL20* expression in AH and its cellular source, we performed different animal model of liver injury representative for some of the key events that occur in AH such as ethanol consumption, fibrosis and endotoxemia. We first tested the effect of ethanol on *Ccl20* hepatic expression. Mice administered with ethanol by gavage did not show increased *Ccl20* hepatic levels (data not shown) while other molecules important in AH such as Fn14 were found increased in this model,[8] suggesting that ethanol itself may not be directly implicated in the regulation of CCL20. We next investigated if CCl_4 administration or LPS induced *Ccl20* hepatic expression. We found that CCl_4 and LPS significantly increased *Ccl20* hepatic gene expression ($p<0.05$) (Figure 4A). Importantly, mice treated with a combination of CCl_4 and LPS resulted in a strong increase of *Ccl20* hepatic expression, as compared to mice treated with LPS, CCl_4 and control mice ($p<0.05$, Figure 4A). The extent of liver damage in mice injected with the combination of CCl_4 and LPS was confirmed by multiple approaches that underlined increased collagen deposition, enhanced hepatic gene expression of *Col1a1*, *Tgfβ*, *Icam1*, and *F4/80* and enhanced protein expression of F4/80, CCL20 and ICAM1 (Figure 4B and 4C).

Macrophages are the main cell source of hepatic CCL20 in LPS-induced liver injury

In order to identify the main cell source of CCL20 in the injured liver, different hepatic cell populations were isolated from livers of mice subjected to a model of acute-on-chronic liver injury (CCl_4 plus LPS) and *Ccl20* expression was assessed. As shown in Figure 4D, we identified

macrophages as the hepatic cell type expressing higher levels of *Ccl20* ($p<0.001$), followed by HSCs, T cells and hepatocytes ($p<0.05$ for all as compared to whole liver). Since macrophages were identified as the main hepatic *Ccl20* cell source, and because their activation is a crucial step in liver inflammation and fibrosis, we also explored *Ccl20* production *in vitro* in RAW264 cell line. We found that LPS induced a strong increase in *Ccl20* gene expression in a dose-dependent manner in these cells (Figure 4E).

Silencing CCL20 ameliorates LPS-induced liver injury

Once identified LPS as one of the major inducers of *Ccl20*, we evaluated the effects mediated by CCL20 in LPS-induced liver injury. Mice treated with LPS showed an important increase in ALT, AST and LDH levels, which were markedly reduced in animals pretreated with shRNA specific for CCL20 as compared to control shRNA (Figure 5A). Moreover, LPS induced an important increase in *Ccl20, Nos2, Icam1, Mcp1, Tgfβ and Col1a1* gene expression. Animals treated with CCL20 shRNA showed a marked reduction in *Ccl20* expression at mRNA and protein level, indicating an efficient knockdown by the shRNA treatment (Figure 5B and C). Moreover, we observed a clear decrease of *Nos2, Icam1, Mcp1* and *Tgfβ* gene expression ($p<0.05$) in animals treated with CCL20 shRNA and LPS. *Col1a1* showed also a tendency to decrease (Figure 5B). We also found a reduction of hepatic protein expression of NOS2 and ICAM1 ($p<0.05$) in mice injected with CCL20 shRNA and LPS as compared to the control group (Figure 5C). Furthermore, CCL20 knockdown reduced macrophages and neutrophils hepatic infiltration ($p<0.05$) (Figure 5D) and caspase-8 ($p=0.059$) and caspase-3 ($p=0.077$) cleavage (Figure 5E). These results suggest that CCL20 mediates LPS-induced hepatocellular damage, regulates important genes known to participate in the pathogenesis of AH and modulates the hepatic inflammatory infiltrate.

DISCUSSION

AH is a form of acute-on-chronic liver damage characterized by hepatocellular damage, inflammatory infiltrate and fibrosis. There is a clear need to identify key drivers of this disease to develop new targeted therapies. Here we investigate the potential role of CCL20, a chemokine that was found importantly up-regulated in patients with AH. We performed a translational approach including hepatic and serum studies and molecular-clinical correlations to evaluate the potential role of CCL20 in AH pathogenesis. Because there are no available animal models reproducing all the features of severe AH, we used experimental models of acute, chronic and acute-on-chronic liver injury, which resemble some of the key hallmarks of AH in humans. A new experimental model to induce severe alcohol liver disease in mice has been recently described [9, 10], however its suitability to study AH still needs to be confirmed. Our results strongly suggest that CCL20 is not only a potential biomarker, but also may play a role in the pathogenesis of AH. This conclusion is based on results showing that CCL20 hepatic and serum levels correlate with disease severity and *in vitro* and experimental data showing that CCL20 mediates fibrosis, inflammation and hepatocellular injury. Obviously, these results needs to be further confirmed in a larger cohort of patients and, when available, in experimental models of severe AH.

AH is characterized by an important inflammatory response that mediates the complex interaction among inflammatory cells, hepatocytes and non-parenchymal cells.[5] Here we show a profound up-regulation of CCL20 in patients with AH and its correlation with key clinical features of the disease and short-term mortality, indicating that CCL20 may represent a good biomarker in patients with AH. Nevertheless, the usefulness of CCL20 to predict AH patient's outcome needs to be further confirmed in a larger cohort of patients. Patients with AH commonly show increased gut permeability and bacterial translocation to the liver with the consequent activation of many hepatic cell types and the activation and perpetuation of the hepatic inflammatory and fibrogenic responses.[13, 28, 29, 30, 31] One of the most striking findings of this study is the strong correlation between circulating CCL20 and LPS serum levels, suggesting that hepatic *CCL20* up-regulation may result from increased levels of circulating pathogen-associated molecular patterns that activate macrophages in the injured liver. Supporting this hypothesis, we identified

macrophages and activated HSCs as the main hepatic *Ccl20* cell sources in an experimental model of acute-on-chronic liver injury where we combined fibrosis and endotoxemia in order to reproduce two of the main events that occur in AH. The specific role of LPS in CCL20 induction was further confirmed in animal models of LPS-induced liver damage, where *Ccl20* hepatic levels were strongly up-regulated following LPS administration. These results indicate that increased gut permeability, that typically occurs in cirrhotic and AH patients may result in an increased *CCL20* hepatic expression.

In addition to being a potential biomarker, we also suggest a role for CCL20 in the pathophysiology of AH. CCL20 is well known to mediate recruitment of CCR6 positive cells during liver injury,[25] which are involved in the amplification of the local inflammatory response.[24, 32, 33, 34] Recently, CCR6 has been shown to exert an important role in the modulation of liver inflammation and fibrosis.[24] However, little is known about the direct effects of CCL20 in the injured liver. Although most of the patients included in our study were cirrhotic, the hepatic expression of CCL20 was significantly higher in patients with METAVIR F4 compared to those with mild fibrosis (METAVIR F1-F3), suggesting that CCL20 could be related to fibrogenesis. We provide evidences that CCL20 exert pro-inflammatory and pro-fibrogenic effects in cultured human primary HSCs and enhances ERK-dependent migration in these cells, suggesting a role for this chemokine in the progression of liver fibrosis.

The main limitation to investigate the mechanisms driving liver injury in AH patients is the lack of an appropriate animal model reproducing the key pathophysiological features of AH. For that reason we investigated the induction of CCL20 expression in animal models of acute-on-chronic liver injury. Interestingly, ethanol administration did not induce by itself *Ccl20* hepatic expression. On the contrary, when damaged livers were challenged with an inflammatory insult (LPS), there was a strong induction of hepatic *Ccl20*. Importantly, CCl_4 and LPS showed an additive effect, suggesting that endotoxemia, in the context of liver fibrosis, may enhance the expression of *CCL20*. This observation suggests that ethanol may not be the direct trigger of CCL20 increase and that endotoxemia may have the predominant role in the induction of hepatic *Ccl20*. The most sensitive hepatic cell types to LPS are macrophages, in which LPS promotes

activation, M1 polarization and the burst of inflammatory events [35, 36] and HSCs. Macrophages and in a lesser extent HSCs and other liver cell types were found as the main cell source of *Ccl20* both *in vitro* and in acute-on-chronic (CCl_4+LPS) liver injury model, suggesting that macrophages and activated HSCs are the main cell type responsible for the cascade of events from LPS-TLR4 activation to *Ccl20* induction and consequent worsening of the hepatic inflammation and fibrosis.

In order to confirm that CCL20 mediates the effects of LPS-induced liver injury, we used a specific shRNA to silence *Ccl20* hepatic expression *in vivo*. The knockdown of *Ccl20* reduced AST, ALT and LDH serum levels, determined a reduction of important hepatic pro-inflammatory and pro-fibrogenic genes and proteins and decreased macrophages and neutrophils hepatic infiltration. These results provide new important findings in the cascade of events in response to LPS-induced liver damage, where CCL20 may play an important role inducing both a direct damage on liver cells and/or participating through an indirect manner in the LPS cascade that leads to liver injury, hepatic inflammation and fibrosis. The fact that CCL20 regulates the expression of other well described molecules involved in the pathogenesis of ALD such as MCP1 [18, 37, 38] and TGFβ [18, 39] is an important finding that allow us to include CCL20 into the group of the pro-inflammatory and pro-fibrogenic molecules that participate in the progression and in the pathogenesis of AH.

Understanding the role of cytokines in liver disease and their interaction with inflammatory and resident hepatic cells is of utmost importance to depict the complex inflammatory response that takes place during AH and to define new therapeutic strategies. Our study demonstrates that CCL20 is markedly up-regulated in patients with AH and provides evidences that CCL20 may be an important mediator in LPS-induced liver inflammation, fibrosis, hepatocellular damage and inflammatory cells recruitment and could be used as a new biomarker to determine AH patient's outcome. However, further pre-clinical studies in future models of AH are required to determine if targeting CCL20 is an effective and safe therapeutic strategy to modulate the inflammatory response and liver injury in AH. Moreover, issues regarding CCL20 specificity, modulation of inflammatory cell recruitment and safety will need special attention to evaluate the potential of CCL20 as a therapeutic target in patients with AH.

Acknowledgements: This work was performed in Centre Esther Koplowitz (CEK). We thank for the precious advices and help Dr Pradere JP from Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, NY, USA; and Dr Beraza N. from CIC bioGUNE, Bilbao, Spain. We are also grateful to the Citomics Unit of the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) for the technical help and to Edurne Berra and Encarnacion Pérez-Andrés from the Gene Silencing Platform at CIC bioGUNE for the production of RNAi-inducing constructs.

Conflict of interest statement: We declare that we have no conflict of interest.

Funding: This work was supported by grants from the Instituto de Salud Carlos III (FIS PI041538, FIS PI042380, FIS PI080126 and FIS PI12/01274 to Dr. Bataller, Dr. Caballería, Dr. Ginès, and Dr. Arroyo respectively) and European Commission within its FP7 Cooperation Programme and Cosmetics Europe, HeMiBio HEALTH F5 2010 266777, and the NIH (1U01AA021908-01-33490 to Dr. Sancho-Bru, 1U01AA021908 to Dr. Bataller and U01AA021912 to Dr. Schwabe). S. Affò received a grant from IDIBAPS. D. Rodrigo-Torres received a grant from the Ministerio de Educación, Cultura y Deporte, FPU program. Dr Sancho-Bru is funded by Instituto de Salud Carlos III, Miguel Servet (CP11/00071) and co-financed by Fondo Europeo de Desarrollo Regional (FEDER), Unión Europea, "Una manera de hacer Europa".

REFERENCES

1 Gao B, Bataller R. Alcoholic liver disease: pathogenesis and new therapeutic targets. *Gastroenterology* 2011;**141**:1572-85.
2 Dominguez M, Rincon D, Abraldes JG*, et al.* A new scoring system for prognostic stratification of patients with alcoholic hepatitis. *Am J Gastroenterol* 2008;**103**:2747-56.
3 Lucey MR, Mathurin P, Morgan TR. Alcoholic hepatitis. *N Engl J Med* 2009;**360**:2758-69.
4 Rosa H, Silverio AO, Perini RF*, et al.* Bacterial infection in cirrhotic patients and its relationship with alcohol. *Am J Gastroenterol* 2000;**95**:1290-3.
5 Altamirano J, Bataller R. Alcoholic liver disease: pathogenesis and new targets for therapy. *Nat Rev Gastroenterol Hepatol* 2011;**8**:491-501.
6 Porter HP, Simon FR, Pope CE, 2nd*, et al.* Corticosteroid therapy in severe alcoholic hepatitis. A double-blind drug trial. *N Engl J Med* 1971;**284**:1350-5.
7 Morales-Ibanez O, Dominguez M, Ki SH*, et al.* Human and experimental evidence supporting a role for osteopontin in alcoholic hepatitis. *Hepatology* 2013. doi: 10.1002/hep.26521.
8 Affo S, Dominguez M, Lozano JJ*, et al.* Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis. *Gut* 2012;**62**:452-60.
9 Bertola A, Mathews S, Ki SH*, et al.* Mouse model of chronic and binge ethanol feeding (the NIAAA model). *Nat Protoc* 2013;**8**:627-37.
10 Bertola A, Park O, Gao B. Chronic plus binge ethanol feeding synergistically induces neutrophil infiltration and liver injury: A critical role for E-selectin. *Hepatology* 2013. doi: 10.1002/hep.26419.
11 Fujimoto M, Uemura M, Nakatani Y*, et al.* Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: relation to severity of liver disturbance. *Alcohol Clin Exp Res* 2000;**24**:48S-54S.
12 Fukui H, Brauner B, Bode JC*, et al.* Plasma endotoxin concentrations in patients with alcoholic and non-alcoholic liver disease: reevaluation with an improved chromogenic assay. *J Hepatol* 1991;**12**:162-9.
13 Rao R. Endotoxemia and gut barrier dysfunction in alcoholic liver disease. *Hepatology* 2009;**50**:638-44.
14 Seki E, Schnabl B. Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut. *J Physiol* 2011;**590**:447-58.
15 Schwabe RF, Seki E, Brenner DA. Toll-like receptor signaling in the liver. *Gastroenterology* 2006;**130**:1886-900.
16 Wheeler MD. Endotoxin and Kupffer cell activation in alcoholic liver disease. *Alcohol Res Health* 2003;**27**:300-6.
17 Seki E, De Minicis S, Osterreicher CH*, et al.* TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007;**13**:1324-32.
18 Colmenero J, Bataller R, Sancho-Bru P*, et al.* Hepatic expression of candidate genes in patients with alcoholic hepatitis: correlation with disease severity. *Gastroenterology* 2007;**132**:687-97.
19 Luster AD. Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998;**338**:436-45.
20 Wasmuth HE, Tacke F, Trautwein C. Chemokines in liver inflammation and fibrosis. *Semin Liver Dis* 2010;**30**:215-25.
21 Hieshima K, Imai T, Opdenakker G*, et al.* Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver. Chemotactic activity for lymphocytes and gene localization on chromosome 2. *J Biol Chem* 1997;**272**:5846-53.
22 Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev* 2003;**14**:409-26.
23 Yamauchi K, Akbar SM, Horiike N*, et al.* Increased serum levels of macrophage inflammatory protein-3alpha in chronic viral hepatitis: prognostic importance of macrophage inflammatory protein-3alpha during interferon therapy in chronic hepatitis C. *J Viral Hepat* 2002;**9**:213-20.

24 Hammerich L, Bangen JM, Govaere O*, et al.* Chemokine receptor CCR6-dependent accumulation of gammadelta T-cells in injured liver restricts hepatic inflammation and fibrosis. *Hepatology* 2013. doi: 10.1002/hep.26697.
25 Shimizu Y, Murata H, Kashii Y*, et al.* CC-chemokine receptor 6 and its ligand macrophage inflammatory protein 3alpha might be involved in the amplification of local necroinflammatory response in the liver. *Hepatology* 2001;**34**:311-9.
26 EASL clinical practical guidelines: management of alcoholic liver disease. *J Hepatol* 2012;**57**:399-420.
27 Sancho-Bru P, Juez E, Moreno M*, et al.* Hepatocarcinoma cells stimulate the growth, migration and expression of pro-angiogenic genes in human hepatic stellate cells. *Liver Int* 2009;**30**:31-41.
28 Aoyama T, Paik YH, Seki E. Toll-like receptor signaling and liver fibrosis. *Gastroenterol Res Pract* 2010;**2010**.
29 Guo J, Friedman SL. Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis. *Fibrogenesis Tissue Repair* 2010;**3**:21.
30 Petrasek J, Mandrekar P, Szabo G. Toll-like receptors in the pathogenesis of alcoholic liver disease. *Gastroenterol Res Pract* 2010;**2010**.
31 Roh YS, Seki E. Toll-like receptors in alcoholic liver disease, non-alcoholic steatohepatitis and carcinogenesis. *J Gastroenterol Hepatol* 2013;**28 Suppl 1**:38-42.
32 Chen KJ, Lin SZ, Zhou L*, et al.* Selective recruitment of regulatory T cell through CCR6-CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis. *PLoS One* 2011;**6**:e24671.
33 Karlmark KR, Weiskirchen R, Zimmermann HW*, et al.* Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis. *Hepatology* 2009;**50**:261-74.
34 Oo YH, Banz V, Kavanagh D*, et al.* CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver. *J Hepatol* 2012;**57**:1044-51.
35 Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. *J Immunol* 2008;**181**:3733-9.
36 Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;**3**:23-35.
37 Afford SC, Fisher NC, Neil DA*, et al.* Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis. *J Pathol* 1998;**186**:82-9.
38 Mandrekar P, Ambade A, Lim A*, et al.* An essential role for monocyte chemoattractant protein-1 in alcoholic liver injury: regulation of proinflammatory cytokines and hepatic steatosis in mice. *Hepatology* 2011;**54**:2185-97.
39 Chen WX, Li YM, Yu CH*, et al.* Quantitative analysis of transforming growth factor beta 1 mRNA in patients with alcoholic liver disease. *World J Gastroenterol* 2002;**8**:379-81.

FIGURE LEGENDS

Figure 1. CCL20 hepatic and serum levels in patients with AH. (A) *CCL20* hepatic gene expression in patients with AH (n=32), NASH (n=8), HCV (n=8) and compensated cirrhosis (n=8) compared to normal livers (n=8) (*p<0.005 vs. normal livers, **p <0.001 vs. normal livers, #p <0.001 vs. other groups). (B) CCL20 serum levels (from peripheral blood) in patients with AH (n=49), HCV (n=8), compensated cirrhosis (n=15) and healthy controls (n=8) (*p<0.005 vs.

controls, **p<0.001 vs. controls, #p<0.001 vs. other groups). (C) Correlation between *CCL20* hepatic gene expression and CCL20 serum levels in patients with AH (n=32) (p=0.03).

Figure 2. CCL20 expression and correlation with clinical features of AH. (A) Correlation between *CCL20* hepatic gene expression and MELD score in patients with AH (n=32) (p<0.0001); (B) *CCL20* hepatic gene expression in patients with AH and METAVIR F4 (patients with cirrhosis, n=27) and METAVIR F1-3 (patients without cirrhosis, n=5) (p=0.01); (C) Comparison of *CCL20* hepatic gene expression and the severity of portal hypertension in patients with AH (severe portal hypertension [HVPG>20mmHg] n=12 and non-severe portal hypertension [HVPG<20mmHg] n=20; p=0.008); (D) Correlation between CCL20 and LPS serum levels in patients with AH (n=49) (p<0.0001). (E) Kaplan-Meier curve showing 90-days mortality according to *CCL20* hepatic gene expression. A value of 80-fold expression ($2^{-\Delta\Delta Ct}$) was identified as the cut-off value with best sensitivity and specificity to define patients with low (≤80-fold) and high (>80-fold) *CCL20* gene expression (p=0.01); (F) Kaplan-Meier curve showing 90-days mortality according to CCL20 serum levels in patients with AH. A value of 260 pg/ml was identified as the cut-off with better sensitivity and specificity to define patients with low (≤260 pg/ml) or high (>260 pg/ml) circulating CCL20 serum levels (p=0.03).

Figure 3. CCL20 production and CCL20 effects on HSCs. (A) *CCL20* gene expression in HSCs incubated with LPS 1μg/ml, TNFα 1ng/ml and IL1β20 ng/ml for 24 hours. (B) HSCs were incubated with CCL20 250ng/ml and 1 μg/ml for 24 and 48 hours respectively. mRNA expression was determined by quantitative real-time PCR and was expressed as fold vs. basal (*p<0.05 respect basal). (C) Effects of CCL20 on HSCs migration were evaluated using a Boyden Chamber. Both CCL20 250ng/ml and PDGF 20ng/ml (used as positive control) increased HSCs cell migration, expressed as mean of migrated cells respect control (*p<0.005). Representative pictures of Giemsa-positive migrated cells (X400 magnification) are also shown for control, CCL20 250ng/ml and PDGF 20ng/ml stimulated cells in presence or absence of 10μM U0126, a specific MEK1/2 inhibitor. (D) Representative Western blot of time-course stimulation of HSCs with CCL20 250ng/ml. CCL20 induced a transient ERK phosphorylation. (E) Quantification of the number of

migrated HSCs incubated with CCL20 250ng/ml in the presence or absence of U0126 10μM (*p<0.005 vs. vehicle; **p=0.014 vs. CCL20 stimulated cells).

Figure 4. *Ccl20* hepatic expression in animal models of liver injury and CCL20 cell source. (A) Hepatic *Ccl20* gene expression in mice treated with CCl_4 (n=6), LPS (n=6) and CCl_4 plus LPS (n=12) (see *Supplementary Material*) (*p<0.05 respect control; **p<0.05 respect control and other groups). (B) Representative images of Sirius Red staining in liver of (i) control (x200 magnification) and (ii) CCl_4 plus LPS-treated mice (x200 magnification) and representative images of F4/80 and CCL20 immunohistochemistry in liver of (i) control and (ii) CCl_4 plus LPS-treated mice (x200 magnification). (C) Hepatic *Col1a1*, *Tgfβ*, *F4/80* and *Icam1* gene expression in mice administered CCl_4 plus LPS (*p<0.05) and representative Western blot of hepatic ICAM1 protein expression and quantification in mice treated with CCl_4 plus LPS compared to control group (*p<0.05). GAPDH has been used as endogenous control (D) *Ccl20* mRNA levels in vitamin A+ HSCs (VitA+), neutrophils (Ly6G+), hepatocytes (Hep), T cells (CD3+), total HSCs (HSCs) and macrophages (F4/80+) isolated from liver of mice administered CCl_4 plus LPS (*p<0.05 respect control, **p<0.01 respect other cell types); as control to normalize results we used whole liver samples from mice treated with CCl_4 plus LPS. (E) *Ccl20* gene expression in RAW264 cells incubated with LPS 10ng/ml, 100ng/ml and 1μg/ml for 24 hours (*p<0.05).

Figure 5. CCL20 mediates LPS-induced liver damage. (A) AST, ALT and LDH serum levels in mice treated with control shRNA (shCtrl) (n=6) or CCL20 shRNA (shCCL20) (n=6) and LPS (see *Supplementary Material*) (*p<0.05 respect vehicle; **p<0.05 respect control shRNA); (B) Hepatic *Ccl20*, *Nos2*, *Icam1*, *Mcp1, Tgfβ* and *Col1a1* gene expression in mice treated with control shRNA (n=6) or CCL20 shRNA (n=6) and LPS (*p<0.05 respect vehicle; **p<0.05 respect control shRNA). (C) Representative Western blot and quantification of hepatic CCL20 and representative pictures of CCL20 immunohistochemistry in liver of mice injected with (i) control shRNA and LPS and (ii) CCL20 shRNA and LPS. Representative Western blots and protein expression quantification of NOS2 and ICAM1 in liver of mice treated with control shRNA or CCL20 shRNA and LPS. GAPDH has been used as endogenous control (*p<0.05 respect control shRNA). (D) Representative F4/80 and MPO immunostainings of liver sections of control shRNA or CCL20 shRNA and LPS treatment

(x200 magnification). Quantification of positive-stained areas is shown in the graphs (*$p<0.05$ respect control shRNA). (E) Representative Western blots of total and cleaved caspase-8 and caspase-3 in liver of mice treated with control shRNA or CCL20 shRNA and LPS. Caspase cleavage is represented as ratio of cleaved caspase vs. total caspase compared to control group.

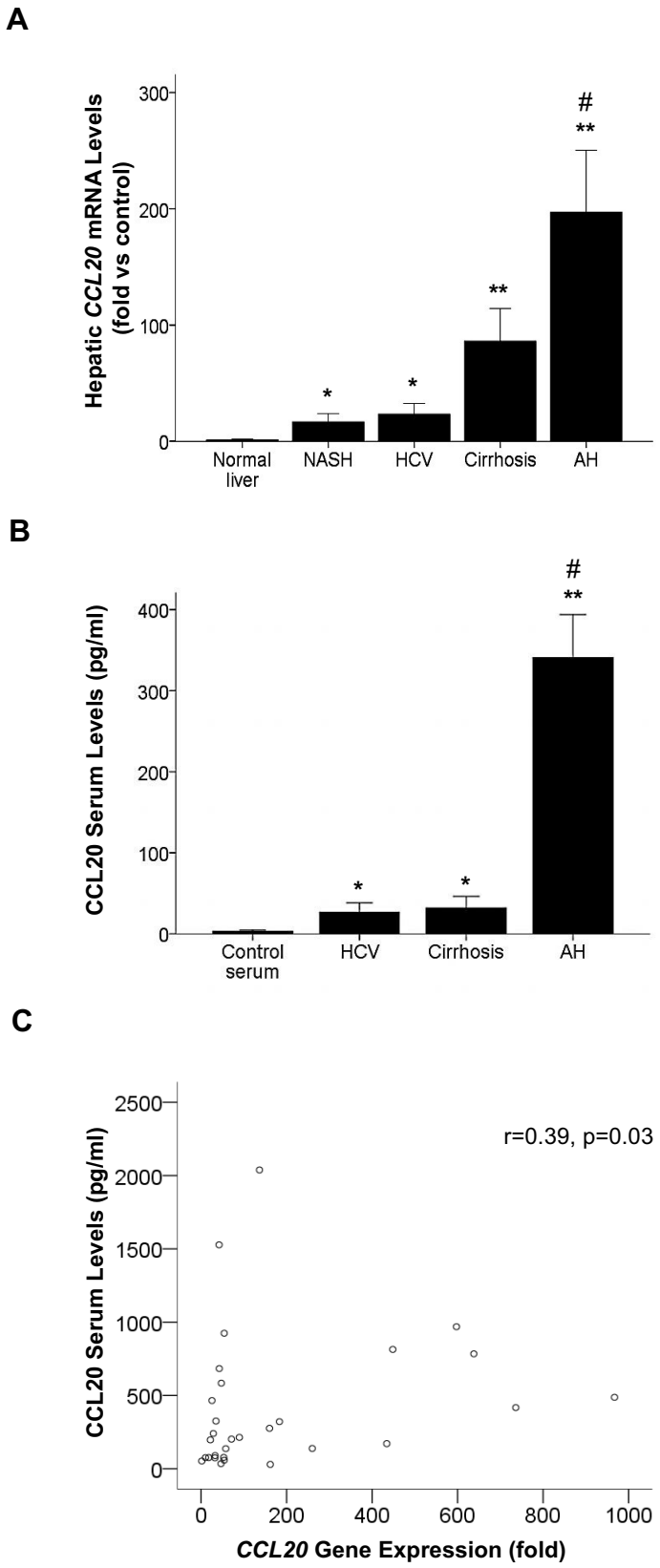

A
Hepatic CCL20 mRNA Levels (fold vs control)
300
200
100
0
#
**
**
*
*
Normal liver
NASH
HCV
Cirrhosis
AH
B
CCL20 Serum Levels (pg/ml)
400
300
200
100
0
#
**
*
*
Control serum
HCV
Cirrhosis
AH
C
CCL20 Serum Levels (pg/ml)
2500
2000
1500
1000
500
0
r=0.39, p=0.03
0
200
400
600
800
1000
CCL20 Gene Expression (fold)

Figure 1

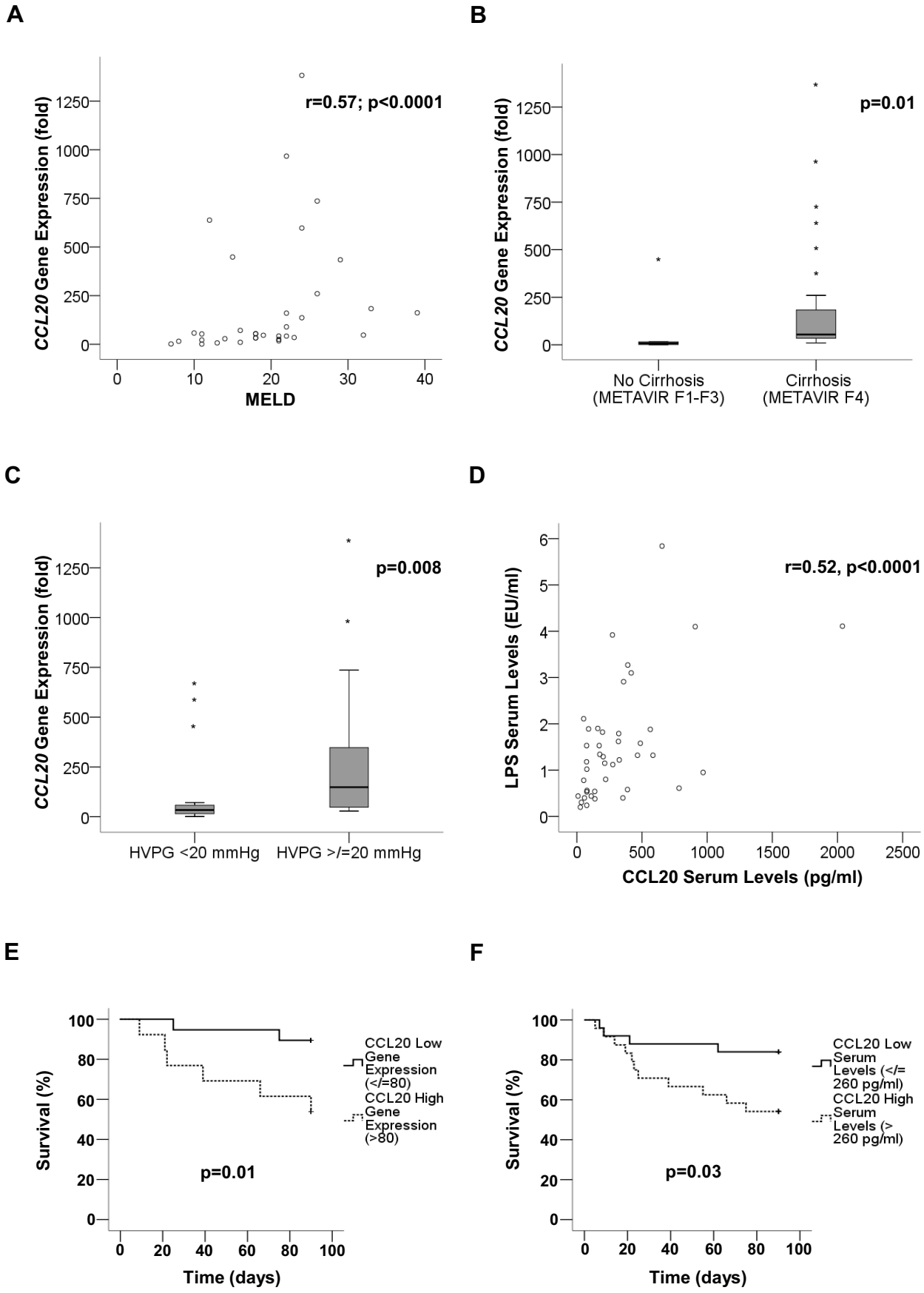

A
CCL20 Gene Expression (fold)
r=0.57; p<0.0001
MELD
B
CCL20 Gene Expression (fold)
p=0.01
No Cirrhosis (METAVIR F1-F3)
Cirrhosis (METAVIR F4)
C
CCL20 Gene Expression (fold)
p=0.008
HVPG <20 mmHg
HVPG >/=20 mmHg
D
LPS Serum Levels (EU/ml)
r=0.52, p<0.0001
CCL20 Serum Levels (pg/ml)
E
Survival (%)
p=0.01
CCL20 Low Gene Expression (</=80)
CCL20 High Gene Expression (>80)
Time (days)
F
Survival (%)
p=0.03
CCL20 Low Serum Levels (</= 260 pg/ml)
CCL20 High Serum Levels (> 260 pg/ml)
Time (days)

Figure 2

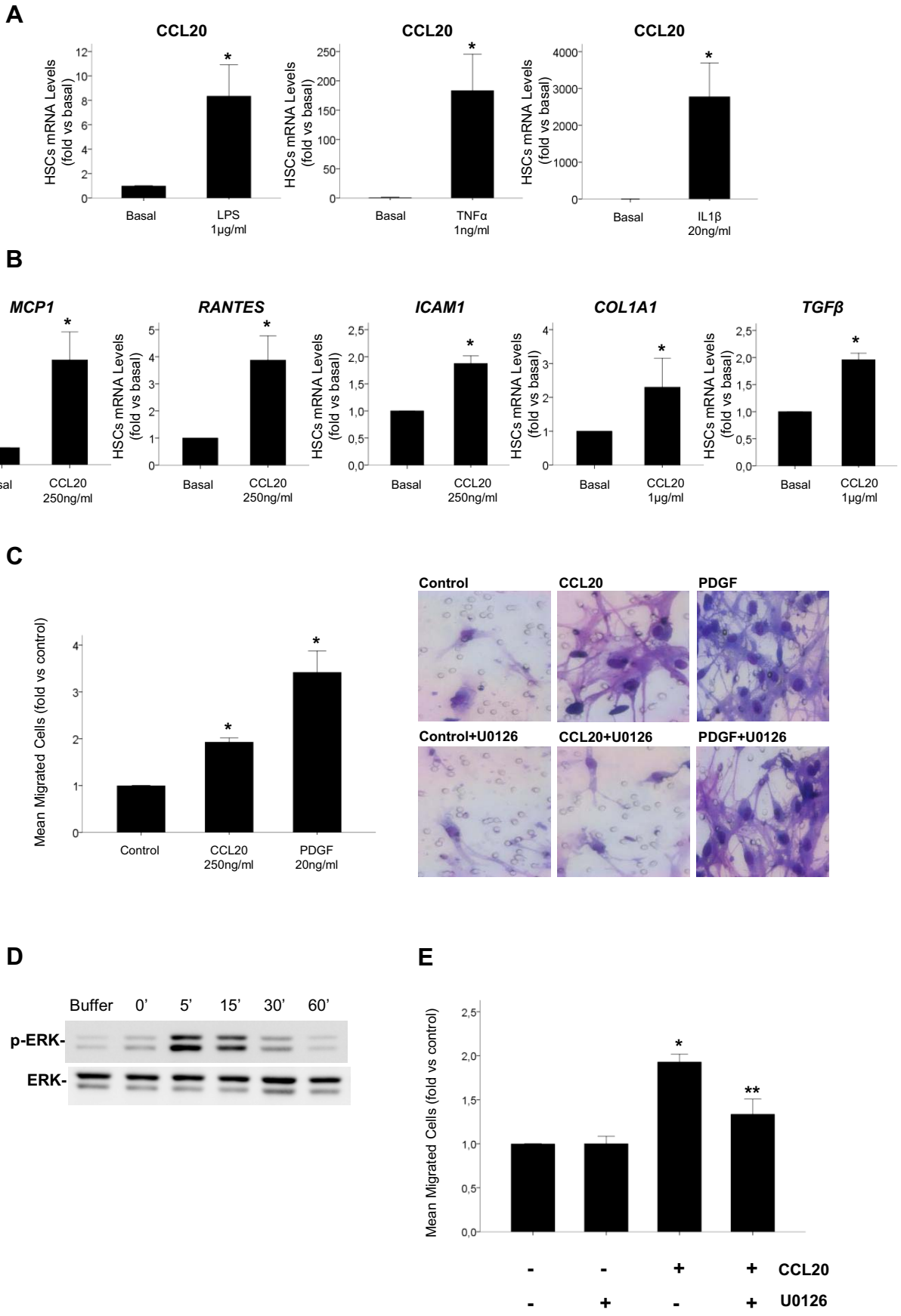

A
CCL20
HSCs mRNA Levels (fold vs basal)
Basal
LPS 1µg/ml
CCL20
Basal
TNFα 1ng/ml
CCL20
Basal
IL1β 20ng/ml
B
MCP1
RANTES
ICAM1
COL1A1
TGFβ
Basal
CCL20 250ng/ml
CCL20 1µg/ml
C
Mean Migrated Cells (fold vs control)
Control
CCL20 250ng/ml
PDGF 20ng/ml
Control
CCL20
PDGF
Control+U0126
CCL20+U0126
PDGF+U0126
D
Buffer
0'
5'
15'
30'
60'
p-ERK-
ERK-
E
Mean Migrated Cells (fold vs control)
CCL20
U0126

Figure 3

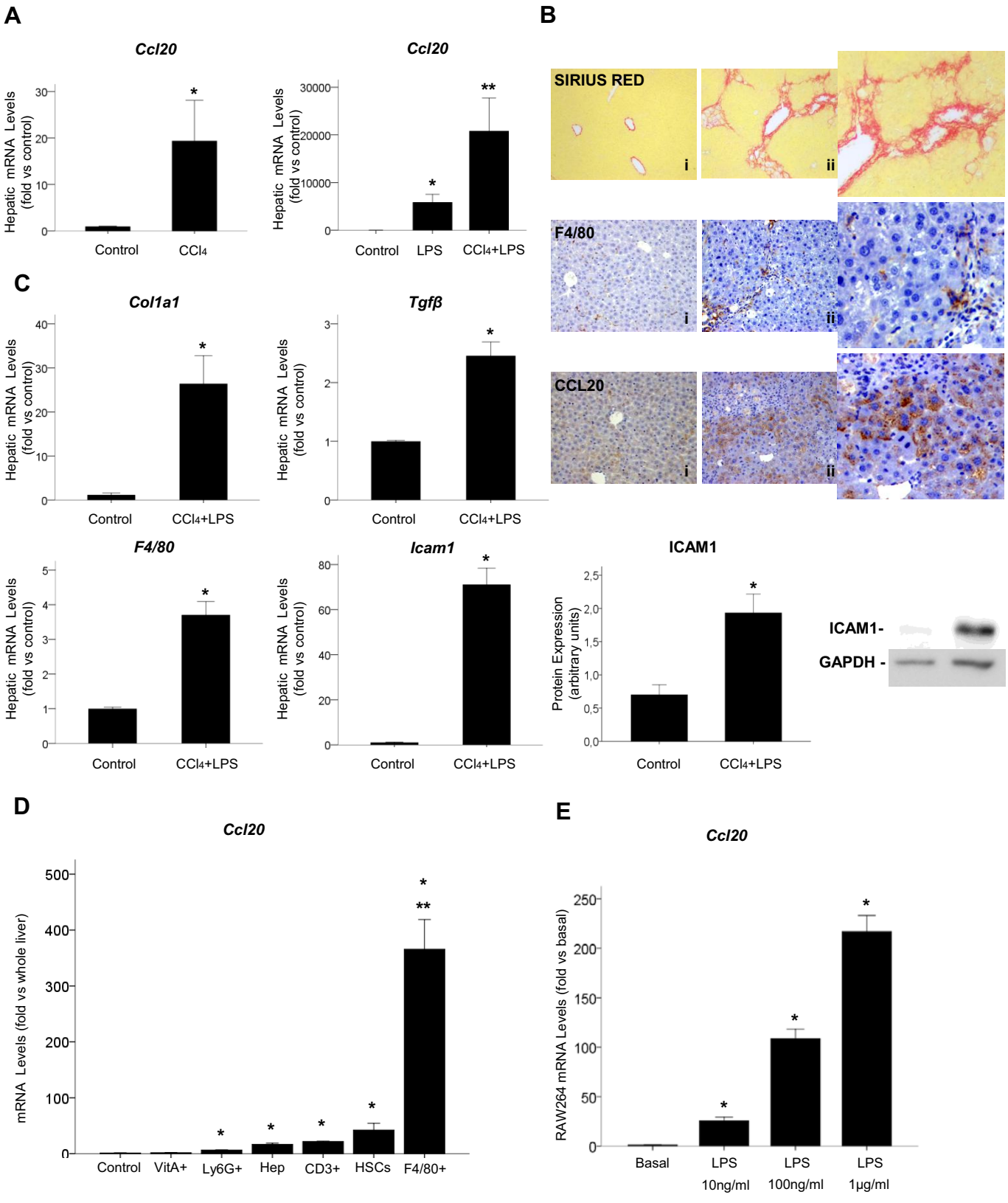

A
Ccl20
Hepatic mRNA Levels (fold vs control)
Control
CCl4
Ccl20
Control
LPS
CCl4+LPS
B
SIRIUS RED
F4/80
CCL20
i
ii
C
Col1a1
Tgfβ
F4/80
Icam1
ICAM1
Protein Expression (arbitrary units)
ICAM1-
GAPDH -
CCl4+LPS
D
Ccl20
mRNA Levels (fold vs whole liver)
Control
VitA+
Ly6G+
Hep
CD3+
HSCs
F4/80+
E
Ccl20
RAW264 mRNA Levels (fold vs basal)
Basal
LPS 10ng/ml
LPS 100ng/ml
LPS 1µg/ml

Figure 4

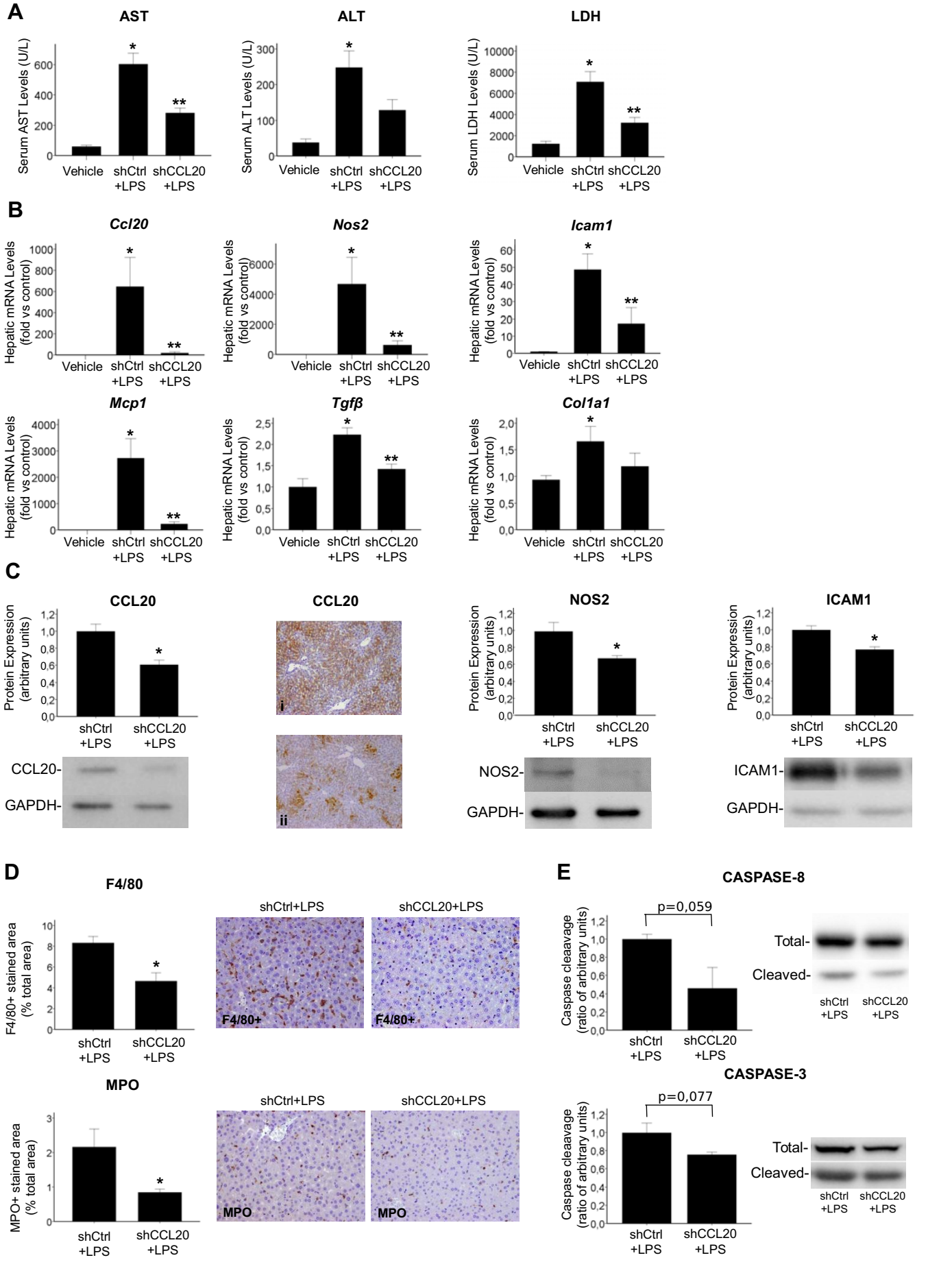

A
AST
ALT
LDH
Serum AST Levels (U/L)
Serum ALT Levels (U/L)
Serum LDH Levels (U/L)
Vehicle
shCtrl +LPS
shCCL20 +LPS
B
Ccl20
Nos2
Icam1
Mcp1
Tgfβ
Col1a1
Hepatic mRNA Levels (fold vs control)
C
CCL20
NOS2
ICAM1
Protein Expression (arbitrary units)
CCL20-
NOS2-
ICAM1-
GAPDH-
i
ii
D
F4/80
F4/80+ stained area (% total area)
shCtrl+LPS
shCCL20+LPS
F4/80+
MPO
MPO+ stained area (% total area)
E
CASPASE-8
CASPASE-3
Caspase cleaavage (ratio of arbitrary units)
p=0,059
p=0,077
Total-
Cleaved-

Figure 5

SUPPLEMENTARY MATERIALS AND METHODS

Histological assessment of polymorphonuclear cells in human biopsies

Liver specimens from patients with AH were formalin-fixed and paraffin-embedded, and slides of 3 μm were stained with hematoxylin-eosin and Masson's trichrome. Regarding polymorphonuclear cells (PMN) assessment, an expert liver pathologist blinded reviewed all biopsies and considered as "mild" PMN infiltration the presence of isolated or a row of few PMNs around one or around a small cluster of 3-4 hepatocytes. (Usually the number of PMNs is less than 15 per focus, and they are difficult to find at low magnification). We considered as "marked" PMN infiltration the presence of PMNs when they were easily recognized at low magnification (x200), and when we observed numerous PMN around damaged hepatocytes (recognized for the presence of ballooning or Mallory-Denk bodies).

RNA isolation and PCR analysis

RNA was isolated from cells and liver tissues using Trizol and following manufacturer's manual instructions (Invitrogen, Carlsbad, CA, USA). RNA from cell-sorted samples was extracted using the QIAGEN RNeasy MICROKit (QIAGEN GmbH, Hilden, Germany). After reverse transcription, mRNA levels were determined by quantitative real-time PCR on an ABI 7900HT cycler (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) using commercial primer-probe pairs (Applied Biosystems) for CCL20, RANTES, COL1A1, F4/80, ICAM1, MCP1, NOS2 and TGFβ. Murine data were normalized to GAPDH and human data were normalized to 18s. The gene expression values were calculated based on the ΔΔCt method. The results were expressed as $2^{-\Delta\Delta Ct}$.

Cell migration assay

Migration assays were performed as previously described using a Boyden Chamber (Neuro Probe, Gaithersburg, MD, USA).[1] Briefly, polycarbonate filters of 8 μm pores (Poretics Corp., Livermore, CA, USA) were coated with 1% gelatin (Sigma-Aldrich, St. Louis, MO, USA). The lower chamber was filled with serum free medium containing 250ng/ml of CCL20 (R&D Systems, Minneapolis, MN, USA) or 20ng/ml of PDGF (R&D Systems) as positive control. As negative control we filled the lower chamber only with serum free medium plus vehicle (PBS containing 0.1% bovine serum albumin). Overnight serum-starved HSCs were trypsinized and placed in the upper chamber ($2x10^4$ cells). After 6 hours of incubation at 37ºC in a 5% CO_2 humidified incubator, cells were fixed with methanol and migrated cells were stained with Giemsa and counted (at x400 magnification). In some experiments cells were pre-incubated with 10μM of U0126, a specific MEK1/2 inhibitor (Cell Signaling Technologies, Danvers, MA, USA) one hour before the incubation with 250ng/ml of CCL20 or with 20ng/ml of PDGF. Each experiment was performed in triplicate, and migration was expressed as fold change respect to control.

Western blot

We performed electrophoresis of protein extracts and subsequent blotting as previously described.[2] Membranes were incubated with primary antibodies against phospho-ERK and total ERK (Cell Signaling Technologies), ICAM1 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), CCL20 (Abcam, Cambridge, UK), NOS2 (Abcam), Caspase-8 and Caspase-3 (Cell Signaling Technologies), GAPDH (Abcam) and with horseradish peroxidase conjugated secondary antibody. GAPDH was used as endogenous control. Proteins were detected by enhanced chemiluminescence (Biological Industries, Beit-Haemek, Israel) and were visualized using Las 4000 Imaging system (GE Healthcare Life Sciences, Piscataway, NJ). The quantification of

the proteins was performed by densitometric analysis using Image GE ImageQuant TL analysis software (GE Healthcare).

Experimental mouse models

To investigate the role of CCL20 in the pathogenesis of liver damage, we performed different animal models. Male Balb/c mice 8 weeks aged were purchased from Charles River (Charles River, l'Arbresle, France). To test the effects of ethanol in CCL20 induction, mice (n=8) were fasted for 8 hours with free access to water and treated with a single dose of 50% ethanol (5g/kg body weight) or water by gavage. Animals were sacrificed 8 hours after gavage as previously described [2]. Next, in order to investigate the effects of LPS in CCL20 induction, mice (n=6) were injected intravenously with lipopolysaccharide (LPS, Sigma-Aldrich) 10mg/kg body weight or saline as control (n=3) and were sacrificed 4 hours after the injection. A chronic liver injury model was performed by injecting mice (n=6) with carbon tetrachloride (CCl_4) intraperitoneally (Sigma-Aldrich; diluted 1:4 in corn oil) at dose of 0.5ml/kg body weight twice per week for a total of 5 injections. Control mice (n=3) were given vehicle (corn oil, Sigma-Aldrich). To mimic the effects of endotoxemia in the context of chronic liver disease we performed a model of acute-on-chronic liver injury. Mice (n=12) were injected with CCl_4 as described in the chronic model and additionally, two days after the last CCl_4 injection, animals were administrated intravenously LPS 10mg/kg body weight and sacrificed 4 hours later. The effects of CCL20 *in vivo* were evaluated in male C57BL/6 mice 8-10 weeks aged. Mice were injected intraperitoneally with 50μg of control shRNA (scrambled sequence) (n=6) or shRNA specific for CCL20 (n=6) complexed with *in vivo* jet-PEI® (Polyplus, Illkirch, France) in a final volume of 400μl. 24 hours later mice were injected intravenously with LPS or saline at dose 2,5mg/kg body weight and immediately after received a second intraperitoneal injection of 50μg of

control shRNA or CCL20 specific shRNA. Mice were sacrificed 24 hours after the last injection. In all animal models, livers were excised and collected.

All animal procedures were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and by the Columbia University Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Assessment of inflammatory injury and fibrosis

Paraffin-embedded liver sections were incubated with primary F4/80 (1:200, Serotec, Oxford, UK), CCL20 (1:200 Santa Cruz Biotechnologies), i NOS (1:150, Abcam), MPO (1:50, Abcam) antibodies overnight at 4°C. After washing, sections were incubated with secondary antibodies (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Finally, the sections were stained with 3,3'-diaminobenzidine (DAB, Dako) and counterstained with hematoxilin. In order to quantify macrophages and neutrophils, sections were visualized at magnification x200 and F4/80-positive and myeloperoxidase (MPO)-positive staining were quantified in 9 different fields for each section by histomorphometry. Results were expressed as % of F4/80-positive and MPO-positive area. To assess the presence of liver fibrosis, liver specimens were stained with Sirius Red (Gurr-BDH Lab Supplies; Poole, England).

Murine hepatic cells isolation, **flow cytometry analysis and cell sorting**

Hepatic cell populations including HSCs and macrophages were isolated by a two-step collagenase-pronase perfusion of livers followed by 17% Nycodenz (Accurate Chemical and Scientific Corporation, Westbury, NY, USA) two-layer discontinuous

density gradient centrifugation as previously described.[3] Cells from liver of mice treated with CCl_4 plus LPS were stained with F4/80-Alexa Fluor 647 (Serotec), CD3-Alexa Fluor 700 and Ly6G-APC (eBioscience, Affymetrix, San Diego, CA, USA). HSCs were purified by vitamin A-based on FACS sorting as previously described.[4, 5] All samples were purified by high speed sorting using a FACSAria cell sorter (Becton, Dickinson and Company, BD, New Jersey, NJ, USA) and immediately lysed in RNA lysis buffer. We also isolated total HSCs as previously described using pronase-collagenase perfusion followed by 9,7% Nycodenz gradient centrifugation.[5, 6]

SUPPLEMENTARY REFERENCES

1 Sancho-Bru P, Juez E, Moreno M*, et al.* Hepatocarcinoma cells stimulate the growth, migration and expression of pro-angiogenic genes in human hepatic stellate cells. *Liver Int* 2009;**30**:31-41.

2 Affò S, Dominguez M, Lozano JJ*, et al.* Transcriptome analysis identifies TNF superfamily receptors as potential therapeutic targets in alcoholic hepatitis. *Gut* 2012;**62**:452-60.

3 Seki E, De Minicis S, Osterreicher CH*, et al.* TLR4 enhances TGF-beta signaling and hepatic fibrosis. *Nat Med* 2007;**13**:1324-32.

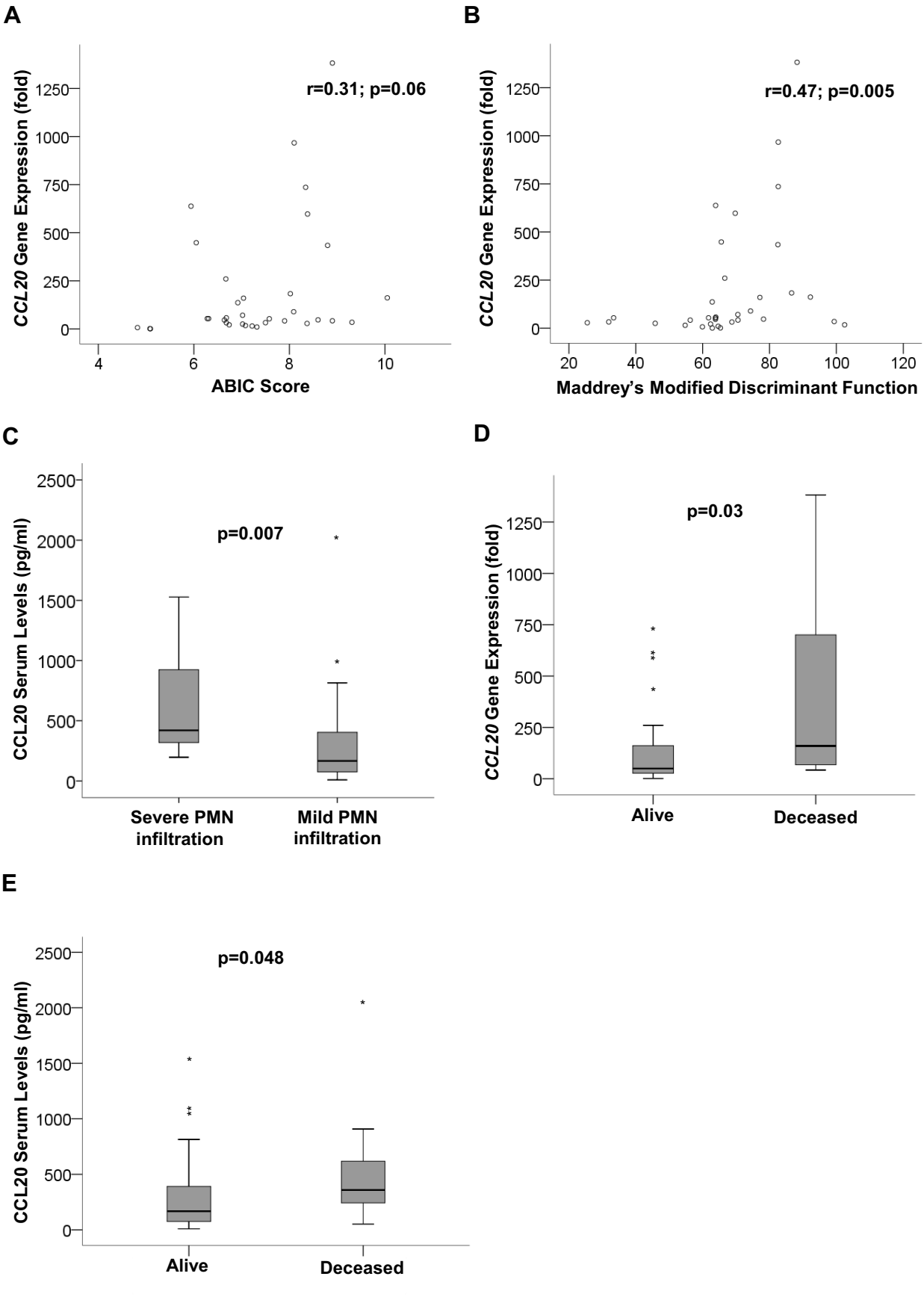
4 Pradere JP, Kluwe J, De Minicis S*, et al.* Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice. *Hepatology* 2013. doi: 10.1002/hep.26429.

5 Troeger JS, Mederacke I, Gwak GY*, et al.* Deactivation of hepatic stellate cells during liver fibrosis resolution in mice. *Gastroenterology* 2012;**143**:1073-83 e22.

6 Mederacke I, Hsu CC, Troeger JS, *et al.* Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis indipendent of its aetiology. Nat Commun. 2013. doi: 10.1038/ncomms3823.

SUPPLEMENTARY FIGURE LEGEND

Supplementary Figure 1. *CCL20* expression and correlations with clinical scores and mortality in patients with AH. (A) Correlation between *CCL20* hepatic gene expression and ABIC score in patients with AH (n=32) (p=0.06); (B) Correlation between *CCL20* hepatic gene expression and Maddrey's modified discriminant function in patients with AH (n=32) (p=0.005); (C) Correlation between CCL20 serum levels and hepatic PMN infiltration (p=0.007); (D,E) Correlation between *CCL20* hepatic gene expression and CCL20 serum levels with mortality at 90-day in patients with AH (p=0.03 [alive n=24, deceased n=8] and p=0.048 [alive n=34, deceased n=15] respectively).



Supplementary Figure 1

DISCUSSION

AH is a form of acute-on-chronic liver damage characterized by hepatocellular damage, inflammatory infiltrate and fibrosis. As largely discussed in previous sections of this thesis, there is a clear need to identify key drivers of AH in order to find new molecular targets for therapy. In fact, the gold-standard therapy for patients with severe AH, remains the use of corticosteroids, proposed as a therapy more than 40 years ago. The lack of significant advances in the management of AH is mostly due to several reasons including: a poor understanding of the pathophysiology of this disease, the lack of experimental models of severe AH, the poor interest in this field from drug companies and the difficulties in concluding clinical trials in patients with active alcohol intake.

As animal models do not accurately mimic the main features of advanced ALD in humans, studies using human samples are of most importance in order to identify new therapeutic targets. Nevertheless, it is important to stress that the pathogenesis of ALD involves several factors and interactions between genes and environment. Therefore, results exclusively based on human samples without taking into consideration environmental factors and without a mechanistic assessment should be considered cautiously. For this reason, it is extremely important to perform translational studies in order to identify new potential targets for therapy in patients with severe ALD, a strategy that we already used in previous studies from our laboratory (Colmenero et al. 2007; Dominguez et al. 2009) to provide new insights to better understand the pathophysiology of AH.

In order to identify genes and pathways differentially regulated in patients with AH, we performed a transcriptome analysis of biopsy-proven patients with clinical, analytical and histological parameters of severe AH. In all patients, liver specimens were obtained using a transjugular approach by an experienced team, which also allowed the measurement of portal pressure. This careful selection of patients led to a homogeneous gene expression pattern throughout our series. It is important to stress that the study was performed in patients with the most severe form of ALD and for this reason, whether the results obtained in our study also apply to patients with moderate forms of ALD is unknown and deserves further investigation.

Using a functional analysis approach, we identified a signature of altered genes differentially expressed in patients with AH and potentially implicated in the pathogenesis of this severe clinical condition. The genes differentially regulated in patients with AH were pooled into seven pathways and, based on the number of deregulated genes belonging to each

pathway, we identified focal adhesion, cell cycle and cytokine–cytokine receptor interaction as the most differentially regulated pathways in AH.

Importantly, some of the data obtained by the microarray analysis and quantitative PCR studies, differed from previous reports describing gene expression changes in rodents exposed to alcohol. This is the case of TNF-α, which has been believed to be one of the major drivers of advanced alcohol liver injury since many years (McClain et al. 1998). In fact, our results using human hepatic samples did not show any difference in TNF-α hepatic expression in patients with AH when compared with control healthy livers. These data confirm the variability between species in reactivity to alcohol exposure and suggest that the current paradigm that TNF-α plays a pivotal role in severe cases of ALD may be revised. Moreover, our results could have important therapeutic implications, since they can explain, at least in part, why TNF-α blocking agents are ineffective in these patients (Naveau et al. 2004).

One of the most remarkable findings of our study was the marked up-regulation of the cytokine–cytokine receptor interaction pathway. We confirmed previous data from our laboratory suggesting that CXC chemokines such as Gro-α and IL-8 may be major players in AH (Colmenero et al. 2007; Dominguez et al. 2009). Furthermore, we provided evidences that some selected CC chemokines such as MCP-1 and CCL20 are strongly up-regulated in these patients. This last finding is in agreement with other studies from our group showing a marked lymphocytic infiltration in patients with AH; whose recruitment is one of the main biological properties of CC chemokines (Dominguez et al. 2009). Importantly, several TNF superfamily receptors such as Fn14, TRAILR1 and FAS were found up-regulated in patients with AH. Surprisingly, none of the ligands of this superfamily including TNF-α, TWEAK, TRAIL and FasL were found differentially regulated. Interestingly, when we increased the number of patients to confirm the microarray data by quantitative PCR, we found that Fn14 was the only receptor exclusively up-regulated in patients with AH, while TRAILR1 was increased in AH and NASH and TNFRSF1 and FAS were exclusively up-regulated in NASH.

In order to further explore the potential role of Fn14 in AH, we first investigated the correlations between Fn14 hepatic gene expression and clinical features of AH. We found that Fn14 hepatic expression was markedly associated with mortality rate at 3 months, suggesting a potential role for this receptor in the pathogenesis of AH. Moreover, we observed that high levels of Fn14 hepatic expression correlated with severity of portal hypertension.

Despite the increase in hepatic gene expression of receptor Fn14, we did not observe any increase in hepatic expression of its ligand, the cytokine TWEAK in our patients with AH. The absence of an up-regulation of TWEAK in AH, suggests that Fn14 may be signaling independently of TWEAK in AH. Moreover, TWEAK serum levels were also found unchanged in patients with AH, thus, suggesting that not only the circulating levels of TWEAK are responsible for increased hepatic Fn14. Actually, Fn14 has been described to be able to signal in a ligand-dependent and also -independent manner when it is ectopically over-expressed in vitro (Winkles 2008). Therefore, based on our results, we hypothesize that Fn14 could act in a TWEAK-independent manner in the liver of patients with AH. Moreover, supporting our hypothesis, we found that in experimental studies in precision-cut liver slices from mice, TWEAK did not induce Fn14 gene expression even if capable of inducing other pro-fibrogenic and pro-inflammatory genes in the liver such as MCP-1, TNF-α, ICAM-1 and TGF-β.

The mechanisms leading to Fn14 up-regulation in AH are unknown. We showed that TGF-β1, a pro-fibrogenic cytokine markedly increased in AH, induced an up-regulation of hepatic Fn14 expression in precision cut liver slices of mice. This effect was not induced by other molecules typically present in AH microenvironment such as TNF-α, TWEAK and LPS, suggesting that fibrogenic, rather than inflammatory mediators, could regulate Fn14 hepatic expression.

Recent data from our laboratory indicate that liver regeneration is impaired in patients with AH and ductular reaction is an important trait of AH histology (Sancho-Bru et al. 2012). Moreover, we showed that the accumulation of hepatic progenitor cells correlates with patient mortality, suggesting a defect in the maturation process that generates functional hepatocytes in these patients and/or an ineffective regenerative attempt that fails to sufficiently regenerate the liver to sustain hepatic function. Fn14 has been described to be expressed in progenitor cells in damaged tissues (Jakubowski et al. 2005) thus, we explored whether Fn14 was also expressed in experimental models characterized by progenitor cell expansion. Indeed, we found a marked expression of Fn14 in mouse models of progenitor cell expansion induced by DDC diet, as well as in experimental models of acute acetaminophen and CCl_4. This finding, together with the co-localization of Fn14 with progenitor cells and newly generated hepatocytes that we found in human samples, suggest a potential role for this receptor in progenitor cell expansion and differentiation in AH.

Importantly, ethanol administration to mice resulted in increased Fn14 expression, suggesting that alcohol consumption can directly stimulate hepatic expression of Fn14 *in vivo*. However, further studies are necessary to address this hypothesis using experimental models of advanced alcohol-induced liver injury. Furthermore, functional studies modifying Fn14 expression in an animal model of AH are required to delineate the precise role of this molecule in AH.

Starting from the transcriptome analysis published in the first article presented in this thesis, we focused our attention on the up-regulation of CC chemokines in patients with AH. Specifically, within the up-regulated chemokines, we selected CCL20, the most up-regulated hepatic cytokine in the whole transcriptome in patients with AH to further investigate its potential role in AH pathogenesis.

AH is characterized by an important inflammatory response that mediates the complex interaction among inflammatory cells, hepatocytes and non-parenchymal cells (Altamirano et al. 2011). With the second study presented in this thesis, we have shown that the up-regulation of CCL20 in patients with AH correlates with key clinical features of the disease such as neutrophil infiltration, grade of fibrosis, endotoxemia and short-term mortality. These results suggest that CCL20 may represent a good biomarker in patients with AH. Nevertheless, the usefulness of CCL20 to predict AH patient's outcome would need to be further investigated in a larger cohort of patients in order to be validated.

In addition to being a potential biomarker in AH, our results suggest a role for CCL20 in the pathophysiology of AH. CCL20 is well known to mediate recruitment of CCR6 positive cells during liver injury (Shimizu et al. 2001) which are involved in the amplification of the local inflammatory response (Karlmark et al. 2009; Chen et al. 2011; Oo et al. 2012; Hammerich et al. 2013). Recently, CCR6 has been shown to exert an important role in the modulation of liver inflammation and fibrosis (Hammerich et al. 2013). However, little is known about the direct effects of CCL20 in the injured liver. Although most of the patients included in our study were cirrhotic, the hepatic expression of CCL20 was significantly higher in patients with METAVIR F4 compared to those with mild fibrosis (METAVIR F1-F3), suggesting that CCL20 could be also related in fibrogenesis. To confirm the fibrogenic properties of CCL20, we incubated human primary HSCs with different concentrations of CCL20 and we provided evidences that CCL20 exerts pro-inflammatory and pro-fibrogenic effects in these cells. Moreover, CCL20 was able to

induce ERK-dependent migration in these cells, suggesting a role for this chemokine in the progression of liver fibrosis.

As previously discussed, patients with AH commonly show increased gut permeability and bacterial translocation to the liver with the consequent activation of many hepatic cell types, which contributes to the activation and perpetuation of the hepatic inflammatory and fibrogenic responses. The two main cell types activated by LPS are macrophages and HSCs, which also are two of the major players in AH, orchestrating inflammatory cell recruitment and production and secretion of pro-inflammatory and pro-fibrogenic cytokines. One of the most striking findings of this study is the strong correlation between circulating CCL20 and LPS serum levels in patients with AH, suggesting that hepatic CCL20 up-regulation may result from increased levels of circulating pathogen-associated molecular patterns that activate macrophages in the injured liver.

The main limitation to investigate the mechanisms driving liver injury in AH patients is the lack of an appropriate animal model reproducing the key pathophysiological features of AH, as largely discussed in this thesis. For that reason, we evaluated the effect of CCL20 in animal models reproducing some of the key features of AH such as inflammation, fibrosis and hepatocellular damage using acute, chronic and acute-on-chronic experimental models of liver injury.

As previously discussed, AH is a form of acute-on-chronic liver failure. This term implies that an acute event triggers the deterioration of the disease and determines the worsening of patient's outcome occurs on a chronic condition. Chronic ALD leads to increased extracellular matrix deposition and liver fibrosis, which we have reproduced in mice with CCl_4 administration, a well-established model of liver fibrosis. On the other hand, the acute events leading to the worsening of the disease remain poorly characterized, but ethanol, endotoxemia and infections, together with genetic and environmental factors are known to trigger the development of AH. To mimic an acute insult, we administered ethanol and LPS separately or in combination to mice after CCl_4 treatment.

Interestingly, ethanol administration did not induce by itself hepatic expression of CCL20, while induced hepatic expression of others molecules including Fn14. On the contrary, when damaged livers were challenged with LPS, there was a strong induction of hepatic chemokine CCL20. Importantly, CCl_4 and LPS showed an additive effect when administered as acute-on-chronic insult, suggesting that endotoxemia, in the context of liver fibrosis, may

enhance the expression of hepatic CCL20. This observation suggests that ethanol may not be the direct trigger of CCL20 increase in patients with AH, and that endotoxemia may have the predominant role in the induction of hepatic CCL20. Taking in consideration these results, we consider that further attention should be taken to systemic mediators of inflammation as may have a profound impact to the pathogenesis and outcome of AH.

As previously stated, the most sensitive hepatic cell types to LPS are macrophages, in which LPS promotes activation, M1 polarization and the burst of inflammatory events (Gordon 2003; Benoit et al. 2008). HSCs have also been shown to be activated in response to LPS. In our study, we found that macrophages and in a lesser extent HSCs and other liver cell types, were the main cell source of CCL20 both *in vitro* and *in vivo* in acute-on-chronic (CCl_4+LPS) liver injury model. It is important to notice that quiescent HSCs barely expressed CCL20, while activated HSCs showed a strongly higher expression of this cytokine when isolated from damaged livers, indicating that CCL20 exerts an important role during fibrogenesis. These results suggest that macrophages and activated HSCs are the main cell type responsible for the cascade of events in liver fibrosis leading from LPS-TLR4 activation to CCL20 induction and consequent worsening of the hepatic inflammation and fibrosis. Supporting this hypothesis, we identified macrophages and activated HSCs as the two main hepatic cell sources of CCL20 in our experimental model of acute-on-chronic liver injury, where we combined fibrosis and endotoxemia. The specific role of LPS in CCL20 induction was further confirmed in animal models of LPS-induced liver damage, where CCL20 hepatic levels were strongly up-regulated following LPS administration. These results indicate that increased gut permeability, that typically occurs in cirrhotic and AH patients, may be responsible for the increased hepatic expression of CCL20 observed in patients with AH.

In order to confirm that CCL20 mediates the effects of LPS-induced liver injury, we used a specific shRNA to knockdown CCL20 hepatic expression *in vivo*. The knockdown of CCL20 reduced AST, ALT and LDH serum levels, as well as important hepatic pro-inflammatory and pro-fibrogenic molecules. Importantly, CCL20 silencing also decreased macrophages and neutrophils hepatic infiltration. These results provide new important findings in the cascade of events in response to LPS-induced liver damage, confirming a role for CCL20 in the promotion of hepatocellular damage and in the expansion of the inflammatory response leading to enhanced hepatic inflammation and fibrosis. The fact that CCL20 regulates the expression of other well described molecules involved in the pathogenesis of ALD such as MCP-1 (Afford et al. 1998; Colmenero et al. 2007; Mandrekar et al. 2011) and TGF-β (Chen et al. 2002;

Colmenero et al. 2007) is an important finding that allow us to include CCL20 into the group of the pro-inflammatory and pro-fibrogenic molecules that participate in the progression and in the pathogenesis of AH.

Our results strongly suggest that CCL20 is not only a potential biomarker, but also plays a role in the pathogenesis of AH. Understanding the role of cytokines in liver disease and their interaction with inflammatory and resident hepatic cells is of utmost importance to depict the complex inflammatory response that takes place during AH and to define new therapeutic strategies. However, further pre-clinical studies in future models of AH will be required to determine if targeting CCL20 is an effective and safe therapeutic strategy to modulate the inflammatory response and liver injury in AH. Moreover, issues regarding CCL20 specificity, modulation of inflammatory cell recruitment and safety will need special attention to evaluate the potential of CCL20 as a therapeutic target in patients with AH.

In conclusion, this thesis includes two studies leading to the identification of new potential targets for therapy in AH. With the first study we aimed to perform a transcriptome analysis in order to identify a pattern of genes differentially regulated in patients with severe AH. The identification of this pattern of genes allowed the identification of new potential targets for the treatment of AH, confirmed previous results, provided new data against old paradigms in AH and, most importantly, furnished new insights for the study of the physiopathology of AH. The identification of Fn14 and CCL20 as new potential targets for therapy in AH and their correlations with key hallmarks of the disease such as ethanol consumption, fibrosis, progenitor cells expansion and endotoxemia underline the complexity of this disease and the crosstalk between many mediators that occurs in AH.

We think that the data presented in this thesis can contribute to delineate the role of Fn14 and CCL20 in AH. Moreover, our translational approach provides new important insights and a useful resource for the study of the pathogenesis of this severe disease.

CONCLUSIONS

The main conclusions obtained as result of the studies presented in this thesis are:

1. AH is characterized by a specific pattern of differentially regulated genes. A functional analysis of the gene expression profile showed the deregulation of several pathways potentially implicated in the pathogenesis of AH such as cytokine-cytokine receptor interaction.

2. Within cytokine-cytokine receptor interaction pathway, Fn14 is the only receptor belonging to TNF superfamily to be exclusively up-regulated in patients with AH, and its expression is associated with disease severity and mortality.

3. Fn14 is up-regulated in experimental models of progenitor cell expansion and co-expressed with Ep-CAM in livers of AH patients, suggesting that may regulate ductular reaction expansion.

4. Fn14 hepatic expression is regulated by ethanol and pro-fibrogenic factors suggesting that alcohol abuse together with profibrogenic mediators may both be directly responsible for the induction of Fn14 expression in ALD.

5. Transcriptome analysis identified CCL20 as the most up-regulated cytokine in the liver of patients with AH. Hepatic expression and serum levels of CCL20 are elevated in patients with AH and are associated with key clinical features of the disease suggesting that besides playing a role in AH pathogenesis, it may also be a non-invasive biomarker.

6. Macrophages and hepatic stellate cells are the main CCL20-producing cell types in experimental models of acute-on-chronic liver injury.

7. CCL20 exerts pro-inflammatory and pro-fibrogenic effects in primary human HSCs and macrophages, suggesting that CCL20 may participate in liver disease in an autocrine or paracrine manner.

8. CCL20 mediates LPS-induced liver injury by promoting hepatocellular apoptosis, expression of pro-inflammatory and pro-fibrogenic mediators and by enhancing macrophages and neutrophils infiltrate recruitment.

By performing a translational study we identified several key pathways deregulated in patients with severe AH. The identification of Fn14 and CCL20 as potential molecular drivers of AH provides new insights for understanding the pathogenesis of this severe disease and links major hallmarks of ALD such as alcohol intake, fibrosis, inflammation and liver regeneration. Our data suggest that cytokines and cytokine-receptor pathway could represent new potential targets for therapy in patients with severe forms of ALD.

BIBLIOGRAPHY

Acosta-Rodriguez, E. V., et al. (2007). "Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells." Nat Immunol **8**(9): 942-9.

Adachi, M. and D. A. Brenner (2005). "Clinical syndromes of alcoholic liver disease." Dig Dis **23**(3-4): 255-63.

Ahlenstiel, G., et al. (2009). "Natural killer cells are polarized toward cytotoxicity in chronic hepatitis C in an interferon-alfa-dependent manner." Gastroenterology **138**(1): 325-35 e1-2.

Albano, E. (2012). "Role of adaptive immunity in alcoholic liver disease." Int J Hepatol **2012**: 893026.

Albano, E. and M. Vidali (2009). "Immune mechanisms in alcoholic liver disease." Genes Nutr **5**(2): 141-7.

Altamirano, J. and R. Bataller (2011). "Alcoholic liver disease: pathogenesis and new targets for therapy." Nat Rev Gastroenterol Hepatol **8**(9): 491-501.

Altamirano, J., et al. (2012). "Acute kidney injury is an early predictor of mortality for patients with alcoholic hepatitis." Clin Gastroenterol Hepatol **10**(1): 65-71 e3.

Amadei, B., et al. (2010). "Activation of natural killer cells during acute infection with hepatitis C virus." Gastroenterology **138**(4): 1536-45.

Arjona, A., et al. (2004). "Circadian rhythms of granzyme B, perforin, IFN-gamma, and NK cell cytolytic activity in the spleen: effects of chronic ethanol." J Immunol **172**(5): 2811-7.

Arthur, M. J. (2000). "Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis." Am J Physiol Gastrointest Liver Physiol **279**(2): G245-9.

Ashkenazi, A. and V. M. Dixit (1998). "Death receptors: signaling and modulation." Science **281**(5381): 1305-8.

Baggiolini, M. (2001). "Chemokines in pathology and medicine." J Intern Med **250**(2): 91-104.

Bamboat, Z. M., et al. (2010). "Conventional DCs reduce liver ischemia/reperfusion injury in mice via IL-10 secretion." J Clin Invest **120**(2): 559-69.

Baroni, G. S., et al. (1996). "Interferon gamma decreases hepatic stellate cell activation and extracellular matrix deposition in rat liver fibrosis." Hepatology **23**(5): 1189-99.

Barreiro, L. B. and L. Quintana-Murci (2009). "From evolutionary genetics to human immunology: how selection shapes host defence genes." Nat Rev Genet **11**(1): 17-30.

Basu, S., et al. (2002). "Molecular cloning and sequencing of 25 different rhesus macaque chemokine cDNAs reveals evolutionary conservation among C, CC, CXC, AND CX3C families of chemokines." Cytokine **18**(3): 140-8.

Bataller, R. and D. A. Brenner (2005). "Liver fibrosis." J Clin Invest **115**(2): 209-18.

Bataller, R., et al. (2003). "NADPH oxidase signal transduces angiotensin II in hepatic stellate cells and is critical in hepatic fibrosis." J Clin Invest **112**(9): 1383-94.

Bautista, A. P. (2002). "Neutrophilic infiltration in alcoholic hepatitis." Alcohol **27**(1): 17-21.

Bautista, A. P. and J. J. Spitzer (1992). "Platelet activating factor stimulates and primes the liver, Kupffer cells and neutrophils to release superoxide anion." Free Radic Res Commun **17**(3): 195-209.

Bendelac, A., et al. (2007). "The biology of NKT cells." Annu Rev Immunol **25**: 297-336.

Benyon, R. C. and M. J. Arthur (2001). "Extracellular matrix degradation and the role of hepatic stellate cells." Semin Liver Dis **21**(3): 373-84.

Benyon, R. C. and J. P. Iredale (2000). "Is liver fibrosis reversible?" Gut **46**(4): 443-6.

Benyon, R. C., et al. (1996). "Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic human liver." Gastroenterology **110**(3): 821-31.

Bertola, A., et al. (2013). "Mouse model of chronic and binge ethanol feeding (the NIAAA model)." Nat Protoc **8**(3): 627-37.

Bertola, A., et al. (2013). "Chronic plus binge ethanol feeding synergistically induces neutrophil infiltration and liver injury in mice: A critical role for E-selectin." Hepatology.

Bodmer, J. L., et al. (2002). "The molecular architecture of the TNF superfamily." Trends Biochem Sci **27**(1): 19-26.

Bonacchi, A., et al. (2001). "Signal transduction by the chemokine receptor CXCR3: activation of Ras/ERK, Src, and phosphatidylinositol 3-kinase/Akt controls cell migration and proliferation in human vascular pericytes." J Biol Chem **276**(13): 9945-54.

Bonecchi, R., et al. (2009). "Chemokines and chemokine receptors: an overview." Front Biosci (Landmark Ed) **14**: 540-51.

Bonner, J. C., et al. (1991). "Differential proliferation of rat lung fibroblasts induced by the platelet-derived growth factor-AA, -AB, and -BB isoforms secreted by rat alveolar macrophages." Am J Respir Cell Mol Biol **5**(6): 539-47.

Borkham-Kamphorst, E., et al. (2004). "Dominant-negative soluble PDGF-beta receptor inhibits hepatic stellate cell activation and attenuates liver fibrosis." Lab Invest **84**(6): 766-77.

Bossen, C., et al. (2006). "Interactions of tumor necrosis factor (TNF) and TNF receptor family members in the mouse and human." J Biol Chem **281**(20): 13964-71.

Brown, S. A., et al. (2006). "TWEAK binding to the Fn14 cysteine-rich domain depends on charged residues located in both the A1 and D2 modules." Biochem J **397**(2): 297-304.

Brown, S. A., et al. (2003). "The Fn14 cytoplasmic tail binds tumour-necrosis-factor-receptor-associated factors 1, 2, 3 and 5 and mediates nuclear factor-kappaB activation." Biochem J **371**(Pt 2): 395-403.

Burt, B. M., et al. (2008). "CD11c identifies a subset of murine liver natural killer cells that responds to adenoviral hepatitis." J Leukoc Biol **84**(4): 1039-46.

Campbell, S., et al. (2006). "Proinflammatory effects of TWEAK/Fn14 interactions in glomerular mesangial cells." J Immunol **176**(3): 1889-98.

Canbay, A., et al. (2004). "Apoptosis: the nexus of liver injury and fibrosis." Hepatology **39**(2): 273-8.

Cao, Q., et al. (2002). "Dilinoleoylphosphatidylcholine decreases acetaldehyde-induced TNF-alpha generation in Kupffer cells of ethanol-fed rats." Biochem Biophys Res Commun **299**(3): 459-64.

Carithers, R. L., Jr., et al. (1989). "Methylprednisolone therapy in patients with severe alcoholic hepatitis. A randomized multicenter trial." Ann Intern Med **110**(9): 685-90.

Carpentier, R., et al. (2011). "Embryonic ductal plate cells give rise to cholangiocytes, periportal hepatocytes, and adult liver progenitor cells." Gastroenterology **141**(4): 1432-8, 1438 e1-4.

Carpino, G., et al. (2004). "Activated hepatic stellate cells in liver cirrhosis. A morphologic and morphometrical study." Ital J Anat Embryol **109**(4): 225-38.

Casey, C. A., et al. (2001). "Alcoholic liver disease and apoptosis." Alcohol Clin Exp Res **25**(5 Suppl ISBRA): 49S-53S.

Casini, A., et al. (1997). "Neutrophil-derived superoxide anion induces lipid peroxidation and stimulates collagen synthesis in human hepatic stellate cells: role of nitric oxide." Hepatology **25**(2): 361-7.

Clouston, A. D., et al. (2007). "Steatosis as a cofactor in other liver diseases: hepatitis C virus, alcohol, hemochromatosis, and others." Clin Liver Dis **11**(1): 173-89, x.

Colmenero, J., et al. (2007). "Hepatic expression of candidate genes in patients with alcoholic hepatitis: correlation with disease severity." Gastroenterology **132**(2): 687-97.

Connolly, M. K., et al. (2009). "In liver fibrosis, dendritic cells govern hepatic inflammation in mice via TNF-alpha." J Clin Invest **119**(11): 3213-25.

Cory, S. and J. M. Adams (2002). "The Bcl2 family: regulators of the cellular life-or-death switch." Nat Rev Cancer **2**(9): 647-56.

Cubero, F. J. and N. Nieto (2006). "Kupffer cells and alcoholic liver disease." Rev Esp Enferm Dig **98**(6): 460-72.

Charo, I. F. and R. M. Ransohoff (2006). "The many roles of chemokines and chemokine receptors in inflammation." N Engl J Med **354**(6): 610-21.

Chedid, A., et al. (1994). "Phospholipid antibodies in alcoholic liver disease." Hepatology **20**(6): 1465-71.

Chedid, A., et al. (1993). "Cell-mediated hepatic injury in alcoholic liver disease. Veterans Affairs Cooperative Study Group 275." Gastroenterology **105**(1): 254-66.

Chen, A., et al. (2013). "Liver fatty acid binding protein (L-Fabp) modulates murine stellate cell activation and diet-induced nonalcoholic fatty liver disease." Hepatology **57**(6): 2202-12.

Chen, K. J., et al. (2011). "Selective recruitment of regulatory T cell through CCR6-CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis." PLoS One **6**(9): e24671.

Chen, W., et al. (2003). "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3." J Exp Med **198**(12): 1875-86.

Chicheportiche, Y., et al. (1997). "TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis." J Biol Chem **272**(51): 32401-10.

Day, C. P. and O. F. James (1998). "Hepatic steatosis: innocent bystander or guilty party?" Hepatology **27**(6): 1463-6.

De Minicis, S., et al. (2010). "Role and cellular source of nicotinamide adenine dinucleotide phosphate oxidase in hepatic fibrosis." Hepatology **52**(4): 1420-30.

De Minicis, S., et al. (2007). "Gene expression profiles during hepatic stellate cell activation in culture and in vivo." Gastroenterology **132**(5): 1937-46.

Dominguez, M., et al. (2009). "Hepatic expression of CXC chemokines predicts portal hypertension and survival in patients with alcoholic hepatitis." Gastroenterology **136**(5): 1639-50.

Dominguez, M., et al. (2008). "A new scoring system for prognostic stratification of patients with alcoholic hepatitis." Am J Gastroenterol **103**(11): 2747-56.

Dong, C. (2008). "Regulation and pro-inflammatory function of interleukin-17 family cytokines." Immunol Rev **226**: 80-6.

Duffield, J. S., et al. (2005). "Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair." J Clin Invest **115**(1): 56-65.

Duncan, A. W., et al. (2009). "Stem cells and liver regeneration." Gastroenterology **137**(2): 466-81.

Dunn, W., et al. (2005). "MELD accurately predicts mortality in patients with alcoholic hepatitis." Hepatology **41**(2): 353-8.

Elphick, D. A., et al. (2007). "Spectrum of liver histology in presumed decompensated alcoholic liver disease." Am J Gastroenterol **102**(4): 780-8.

Elsharkawy, A. M., et al. (2005). "The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis." Apoptosis **10**(5): 927-39.

Fallowfield, J. A., et al. (2007). "Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis." J Immunol **178**(8): 5288-95.

Farfan Labonne, B. E., et al. (2009). "Acetaldehyde-induced mitochondrial dysfunction sensitizes hepatocytes to oxidative damage." Cell Biol Toxicol **25**(6): 599-609.

Fava, G., et al. (2008). "Leptin enhances cholangiocarcinoma cell growth." Cancer Res **68**(16): 6752-61.

Feng, S. L., et al. (2000). "The Fn14 immediate-early response gene is induced during liver regeneration and highly expressed in both human and murine hepatocellular carcinomas." Am J Pathol **156**(4): 1253-61.

Fernandez, M., et al. (2009). "Angiogenesis in liver disease." J Hepatol **50**(3): 604-20.

Fischer, D., et al. (2004). "Switching mature retinal ganglion cells to a robust growth state in vivo: gene expression and synergy with RhoA inactivation." J Neurosci **24**(40): 8726-40.

Forkert, P. G., et al. (1991). "Distribution of cytochrome CYP2E1 in murine liver after ethanol and acetone administration." Carcinogenesis **12**(12): 2259-68.

Forrest, E. H., et al. (2005). "Analysis of factors predictive of mortality in alcoholic hepatitis and derivation and validation of the Glasgow alcoholic hepatitis score." Gut **54**(8): 1174-9.

Friedman, S. L. (2008). "Hepatic stellate cells: protean, multifunctional, and enigmatic cells of the liver." Physiol Rev **88**(1): 125-72.

Fujimoto, M., et al. (2000). "Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: relation to severity of liver disturbance." Alcohol Clin Exp Res **24**(4 Suppl): 48S-54S.

Furuyama, K., et al. (2010). "Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine." Nat Genet **43**(1): 34-41.

Gabele, E., et al. (2003). "Liver fibrosis: signals leading to the amplification of the fibrogenic hepatic stellate cell." Front Biosci **8**: d69-77.

Gao, B. (2005). "Cytokines, STATs and liver disease." Cell Mol Immunol **2**(2): 92-100.

Gao, B. and R. Bataller (2011). "Alcoholic liver disease: pathogenesis and new therapeutic targets." Gastroenterology **141**(5): 1572-85.

Gao, B., et al. (2008). "Liver: An organ with predominant innate immunity." Hepatology **47**(2): 729-36.

Gao, B., et al. (2009). "Liver natural killer and natural killer T cells: immunobiology and emerging roles in liver diseases." J Leukoc Biol **86**(3): 513-28.

Gao, B., et al. (2011). "Innate immunity in alcoholic liver disease." Am J Physiol Gastrointest Liver Physiol **300**(4): G516-25.

Gasque, P. (2004). "Complement: a unique innate immune sensor for danger signals." Mol Immunol **41**(11): 1089-98.

George, J., et al. (1999). "In vivo inhibition of rat stellate cell activation by soluble transforming growth factor beta type II receptor: a potential new therapy for hepatic fibrosis." Proc Natl Acad Sci U S A **96**(22): 12719-24.

Gines, P., et al. (2004). "Management of cirrhosis and ascites." N Engl J Med **350**(16): 1646-54.

Girgenrath, M., et al. (2006). "TWEAK, via its receptor Fn14, is a novel regulator of mesenchymal progenitor cells and skeletal muscle regeneration." EMBO J **25**(24): 5826-39.

Glassner, A., et al. (2012). "NK cells from HCV-infected patients effectively induce apoptosis of activated primary human hepatic stellate cells in a TRAIL-, FasL- and NKG2D-dependent manner." Lab Invest **92**(7): 967-77.

Glenney, G. W. and G. D. Wiens (2007). "Early diversification of the TNF superfamily in teleosts: genomic characterization and expression analysis." J Immunol **178**(12): 7955-73.

Glimcher, L. H. and K. M. Murphy (2000). "Lineage commitment in the immune system: the T helper lymphocyte grows up." Genes Dev **14**(14): 1693-711.

Gressner, A. M. and R. Weiskirchen (2006). "Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets." J Cell Mol Med **10**(1): 76-99.

Guicciardi, M. E. and G. J. Gores (2005). "Apoptosis: a mechanism of acute and chronic liver injury." Gut **54**(7): 1024-33.

Guo, J. and S. L. Friedman (2010). "Toll-like receptor 4 signaling in liver injury and hepatic fibrogenesis." Fibrogenesis Tissue Repair **3**: 21.

Hacker, G., et al. (2002). "Activation of the immune system by bacterial CpG-DNA." Immunology **105**(3): 245-51.

Hammerich, L., et al. (2013). "Chemokine receptor CCR6-dependent accumulation of gammadelta T-cells in injured liver restricts hepatic inflammation and fibrosis." Hepatology.

Hammerich, L., et al. (2011). "Role of IL-17 and Th17 cells in liver diseases." Clin Dev Immunol **2011**: 345803.

Han, S., et al. (2003). "TNF-related weak inducer of apoptosis receptor, a TNF receptor superfamily member, activates NF-kappa B through TNF receptor-associated factors." Biochem Biophys Res Commun **305**(4): 789-96.

Harada, N., et al. (2002). "Pro-inflammatory effect of TWEAK/Fn14 interaction on human umbilical vein endothelial cells." Biochem Biophys Res Commun **299**(3): 488-93.

Helman, R. A., et al. (1971). "Alcoholic hepatitis. Natural history and evaluation of prednisolone therapy." Ann Intern Med **74**(3): 311-21.

Henderson, N. C. and J. P. Iredale (2007). "Liver fibrosis: cellular mechanisms of progression and resolution." Clin Sci (Lond) **112**(5): 265-80.

Hernandez-Gea, V. and S. L. Friedman (2010). "Pathogenesis of liver fibrosis." Annu Rev Pathol **6**: 425-56.

Hieshima, K., et al. (1997). "Molecular cloning of a novel human CC chemokine liver and activation-regulated chemokine (LARC) expressed in liver. Chemotactic activity for lymphocytes and gene localization on chromosome 2." J Biol Chem **272**(9): 5846-53.

Higuchi, H. and G. J. Gores (2003). "Mechanisms of liver injury: an overview." Curr Mol Med **3**(6): 483-90.

Hintermann, E., et al. (2010). "CXCL10 promotes liver fibrosis by prevention of NK cell mediated hepatic stellate cell inactivation." J Autoimmun **35**(4): 424-35.

Ho, D. H., et al. (2004). "Soluble tumor necrosis factor-like weak inducer of apoptosis overexpression in HEK293 cells promotes tumor growth and angiogenesis in athymic nude mice." Cancer Res **64**(24): 8968-72.

Holt, A. P., et al. (2009). "Liver myofibroblasts regulate infiltration and positioning of lymphocytes in human liver." Gastroenterology **136**(2): 705-14.

Hritz, I., et al. (2008). "The critical role of toll-like receptor (TLR) 4 in alcoholic liver disease is independent of the common TLR adapter MyD88." Hepatology **48**(4): 1224-31.

Hromas, R., et al. (1997). "Cloning and characterization of exodus, a novel beta-chemokine." Blood **89**(9): 3315-22.

Hsu, W., et al. (2007). "The current immune function of hepatic dendritic cells." Cell Mol Immunol **4**(5): 321-8.

Iimuro, Y., et al. (1997). "Antibodies to tumor necrosis factor alfa attenuate hepatic necrosis and inflammation caused by chronic exposure to ethanol in the rat." Hepatology **26**(6): 1530-7.

Inagaki, Y. and I. Okazaki (2007). "Emerging insights into Transforming growth factor beta Smad signal in hepatic fibrogenesis." Gut **56**(2): 284-92.

Iredale, J. P. (2007). "Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ." J Clin Invest **117**(3): 539-48.

Iredale, J. P., et al. (1996). "Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis." Hepatology **24**(1): 176-84.

Iredale, J. P., et al. (1998). "Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors." J Clin Invest **102**(3): 538-49.

Iredale, J. P., et al. (1992). "Human hepatic lipocytes synthesize tissue inhibitor of metalloproteinases-1. Implications for regulation of matrix degradation in liver." J Clin Invest **90**(1): 282-7.

Issa, R., et al. (2001). "Apoptosis of hepatic stellate cells: involvement in resolution of biliary fibrosis and regulation by soluble growth factors." Gut **48**(4): 548-57.

Issa, R., et al. (2004). "Spontaneous recovery from micronodular cirrhosis: evidence for incomplete resolution associated with matrix cross-linking." Gastroenterology **126**(7): 1795-808.

Ivanov, II, et al. (2006). "The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells." Cell **126**(6): 1121-33.

Jaeschke, H. (2002). "Inflammation in response to hepatocellular apoptosis." Hepatology **35**(4): 964-6.

Jaeschke, H. (2002). "Neutrophil-mediated tissue injury in alcoholic hepatitis." Alcohol **27**(1): 23-7.

Jakubowski, A., et al. (2005). "TWEAK induces liver progenitor cell proliferation." J Clin Invest **115**(9): 2330-40.

Janeway, C. A., Jr. and R. Medzhitov (2002). "Innate immune recognition." Annu Rev Immunol **20**: 197-216.

Jeong, W. I., et al. (2008). "Abrogation of the antifibrotic effects of natural killer cells/interferon-gamma contributes to alcohol acceleration of liver fibrosis." Gastroenterology **134**(1): 248-58.

Jeong, W. I., et al. (2006). "STAT1 inhibits liver fibrosis in mice by inhibiting stellate cell proliferation and stimulating NK cell cytotoxicity." Hepatology **44**(6): 1441-51.

Jiao, J., et al. (2011). "Dendritic cell regulation of carbon tetrachloride-induced murine liver fibrosis regression." Hepatology **55**(1): 244-55.

Jung, Y., et al. (2008). "Accumulation of hedgehog-responsive progenitors parallels alcoholic liver disease severity in mice and humans." Gastroenterology **134**(5): 1532-43.

Karlmark, K. R., et al. (2009). "Hepatic recruitment of the inflammatory Gr1+ monocyte subset upon liver injury promotes hepatic fibrosis." Hepatology **50**(1): 261-74.

Kawakita, T., et al. (2005). "Functional expression of TWEAK in human colonic adenocarcinoma cells." Int J Oncol **26**(1): 87-93.

Kendrick, S. F., et al. (2010). "Acetate, the key modulator of inflammatory responses in acute alcoholic hepatitis." Hepatology **51**(6): 1988-97.

Kinnman, N. and C. Housset (2002). "Peribiliary myofibroblasts in biliary type liver fibrosis." Front Biosci **7**: d496-503.

Kmiec, Z. (2001). "Cooperation of liver cells in health and disease." Adv Anat Embryol Cell Biol **161**: III-XIII, 1-151.

Knittel, T., et al. (1999). "Rat liver myofibroblasts and hepatic stellate cells: different cell populations of the fibroblast lineage with fibrogenic potential." Gastroenterology **117**(5): 1205-21.

Krammer, P. H. (2000). "CD95's deadly mission in the immune system." Nature **407**(6805): 789-95.

Krizhanovsky, V., et al. (2008). "Senescence of activated stellate cells limits liver fibrosis." Cell **134**(4): 657-67.

Kruglov, E. A., et al. (2006). "Secretion of MCP-1/CCL2 by bile duct epithelia induces myofibroblastic transdifferentiation of portal fibroblasts." Am J Physiol Gastrointest Liver Physiol **290**(4): G765-71.

Kurose, I., et al. (1997). "Oxidative stress-mediated apoptosis of hepatocytes exposed to acute ethanol intoxication." Hepatology **25**(2): 368-78.

Lasagni, L., et al. (2003). "An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4." J Exp Med **197**(11): 1537-49.

Laskin, C. A., et al. (1990). "Autoantibodies in alcoholic liver disease." Am J Med **89**(2): 129-33.

Laso, F. J., et al. (1997). "Decreased natural killer cytotoxic activity in chronic alcoholism is associated with alcohol liver disease but not active ethanol consumption." Hepatology **25**(5): 1096-100.

Laso, F. J., et al. (2005). "[Immune system and alcoholic liver disease]." Med Clin (Barc) **125**(7): 263-9.

Lemmers, A., et al. (2009). "The interleukin-17 pathway is involved in human alcoholic liver disease." Hepatology **49**(2): 646-57.

Li, M. O., et al. (2006). "Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms." Immunity **25**(3): 455-71.

Locksley, R. M., et al. (2001). "The TNF and TNF receptor superfamilies: integrating mammalian biology." Cell **104**(4): 487-501.

Lotersztajn, S., et al. (2005). "Hepatic fibrosis: molecular mechanisms and drug targets." Annu Rev Pharmacol Toxicol **45**: 605-28.

Louvet, A., et al. (2011). "Cannabinoid CB2 receptors protect against alcoholic liver disease by regulating Kupffer cell polarization in mice." Hepatology **54**(4): 1217-26.

Lowes, K. N., et al. (1999). "Oval cell numbers in human chronic liver diseases are directly related to disease severity." Am J Pathol **154**(2): 537-41.

Lu, Y. C., et al. (2008). "LPS/TLR4 signal transduction pathway." Cytokine **42**(2): 145-51.

Lucey, M. R., et al. (2009). "Alcoholic hepatitis." N Engl J Med **360**(26): 2758-69.

Maddrey, W. C., et al. (1978). "Corticosteroid therapy of alcoholic hepatitis." Gastroenterology **75**(2): 193-9.

Maecker, H., et al. (2005). "TWEAK attenuates the transition from innate to adaptive immunity." Cell **123**(5): 931-44.

Maher, J. J. (2001). "Interactions between hepatic stellate cells and the immune system." Semin Liver Dis **21**(3): 417-26.

Mangan, P. R., et al. (2006). "Transforming growth factor-beta induces development of the T(H)17 lineage." Nature **441**(7090): 231-4.

Mann, D. A. and J. Mann (2008). "Epigenetic regulation of hepatic stellate cell activation." J Gastroenterol Hepatol **23 Suppl 1**: S108-11.

Mann, J., et al. (2009). "MeCP2 controls an epigenetic pathway that promotes myofibroblast transdifferentiation and fibrosis." Gastroenterology **138**(2): 705-14, 714 e1-4.

Mannaerts, I., et al. (2010). "Chronic administration of valproic acid inhibits activation of mouse hepatic stellate cells in vitro and in vivo." Hepatology **51**(2): 603-14.

Marra, F. (2007). "Leptin and liver tissue repair: do rodent models provide the answers?" J Hepatol **46**(1): 12-8.

Marra, F. and C. Bertolani (2009). "Adipokines in liver diseases." Hepatology **50**(3): 957-69.

Marra, F., et al. (1995). "Thrombin stimulates proliferation of liver fat-storing cells and expression of monocyte chemotactic protein-1: potential role in liver injury." Hepatology **22**(3): 780-7.

Marra, F., et al. (1993). "Cultured human liver fat-storing cells produce monocyte chemotactic protein-1. Regulation by proinflammatory cytokines." J Clin Invest **92**(4): 1674-80.

McClain, C. J., et al. (1998). "Tumor necrosis factor and alcoholic liver disease." Alcohol Clin Exp Res **22**(5 Suppl): 248S-252S.

McClain, C. J., et al. (2004). "Recent advances in alcoholic liver disease. IV. Dysregulated cytokine metabolism in alcoholic liver disease." Am J Physiol Gastrointest Liver Physiol **287**(3): G497-502.

McCoy, S. L., et al. (2004). "Activation of RAW264.7 macrophages by bacterial DNA and lipopolysaccharide increases cell surface DNA binding and internalization." J Biol Chem **279**(17): 17217-23.

McCullough, A. J. and J. F. O'Connor (1998). "Alcoholic liver disease: proposed recommendations for the American College of Gastroenterology." Am J Gastroenterol **93**(11): 2022-36.

McFarlane, I. G. (2000). "Autoantibodies in alcoholic liver disease." Addict Biol **5**(2): 141-51.

Meighan-Mantha, R. L., et al. (1999). "The mitogen-inducible Fn14 gene encodes a type I transmembrane protein that modulates fibroblast adhesion and migration." J Biol Chem **274**(46): 33166-76.

Mencin, A., et al. (2009). "Toll-like receptors as targets in chronic liver diseases." Gut **58**(5): 704-20.

Meraviglia, S., et al. (2011). "gammadelta T cells cross-link innate and adaptive immunity in Mycobacterium tuberculosis infection." Clin Dev Immunol **2011**: 587315.

Michalopoulos, G. K. (2007). "Liver regeneration." J Cell Physiol **213**(2): 286-300.

Milani, S., et al. (1990). "Procollagen expression by nonparenchymal rat liver cells in experimental biliary fibrosis." Gastroenterology **98**(1): 175-84.

Miller, A. M., et al. (2011). "Molecular mechanisms of alcoholic liver disease: innate immunity and cytokines." Alcohol Clin Exp Res **35**(5): 787-93.

Mogensen, T. H. (2009). "Pathogen recognition and inflammatory signaling in innate immune defenses." Clin Microbiol Rev **22**(2): 240-73, Table of Contents.

Morales-Ibanez, O., et al. (2013). "Human and experimental evidence supporting a role for osteopontin in alcoholic hepatitis." Hepatology.

Moreira, R. K. (2007). "Hepatic stellate cells and liver fibrosis." Arch Pathol Lab Med **131**(11): 1728-34.

Moreno, M. and R. Bataller (2008). "Cytokines and renin-angiotensin system signaling in hepatic fibrosis." Clin Liver Dis **12**(4): 825-52, ix.

Moreno, M., et al. (2010). "Ghrelin attenuates hepatocellular injury and liver fibrogenesis in rodents and influences fibrosis progression in humans." Hepatology **51**(3): 974-85.

Mosmann, T. R., et al. (1986). "Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins." J Immunol **136**(7): 2348-57.

Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." Nat Rev Immunol **8**(12): 958-69.

Muhanna, N., et al. (2010). "Amelioration of hepatic fibrosis by NK cell activation." Gut **60**(1): 90-8.

Murohisa, G., et al. (2002). "Involvement of platelet-activating factor in hepatic apoptosis and necrosis in chronic ethanol-fed rats given endotoxin." Liver **22**(5): 394-403.

Murphy, F. R., et al. (2002). "Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: implications for reversibility of liver fibrosis." J Biol Chem **277**(13): 11069-76.

Murphy, K. M. and S. L. Reiner (2002). "The lineage decisions of helper T cells." Nat Rev Immunol **2**(12): 933-44.

Murray, P. J. and T. A. Wynn (2011). "Protective and pathogenic functions of macrophage subsets." Nat Rev Immunol **11**(11): 723-37.

Nakayama, M., et al. (2002). "Multiple pathways of TWEAK-induced cell death." J Immunol **168**(2): 734-43.

Nanji, A. A., et al. (1993). "Severity of liver injury in experimental alcoholic liver disease. Correlation with plasma endotoxin, prostaglandin E2, leukotriene B4, and thromboxane B2." Am J Pathol **142**(2): 367-73.

Natori, S., et al. (2001). "Hepatocyte apoptosis is a pathologic feature of human alcoholic hepatitis." J Hepatol **34**(2): 248-53.

Naveau, S., et al. (2004). "A double-blind randomized controlled trial of infliximab associated with prednisolone in acute alcoholic hepatitis." Hepatology **39**(5): 1390-7.

Nelson, R. T., et al. (2001). "Genomic organization of the CC chemokine mip-3alpha/CCL20/larc/exodus/SCYA20, showing gene structure, splice variants, and chromosome localization." Genomics **73**(1): 28-37.

Nieto, N., et al. (2002). "Stimulation and proliferation of primary rat hepatic stellate cells by cytochrome P450 2E1-derived reactive oxygen species." Hepatology **35**(1): 62-73.

Nischalke, H. D., et al. (2004). "Semiquantitative analysis of intrahepatic CC-chemokine mRNas in chronic hepatitis C." Mediators Inflamm **13**(5-6): 357-9.

Oo, Y. H., et al. (2012). "CXCR3-dependent recruitment and CCR6-mediated positioning of Th-17 cells in the inflamed liver." J Hepatol **57**(5): 1044-51.

Ouyang, W., et al. (2008). "The biological functions of T helper 17 cell effector cytokines in inflammation." Immunity **28**(4): 454-67.

Paez, J. and W. R. Sellers (2003). "PI3K/PTEN/AKT pathway. A critical mediator of oncogenic signaling." Cancer Treat Res **115**: 145-67.

Paik, Y. H., et al. (2003). "Toll-like receptor 4 mediates inflammatory signaling by bacterial lipopolysaccharide in human hepatic stellate cells." Hepatology **37**(5): 1043-55.

Palsson-McDermott, E. M. and L. A. O'Neill (2004). "Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4." Immunology **113**(2): 153-62.

Park, O., et al. (2009). "Diverse roles of invariant natural killer T cells in liver injury and fibrosis induced by carbon tetrachloride." Hepatology **49**(5): 1683-94.

Paronetto, F. (1993). "Immunologic reactions in alcoholic liver disease." Semin Liver Dis **13**(2): 183-95.

Patsenker, E., et al. (2011). "Cannabinoid receptor type I modulates alcohol-induced liver fibrosis." Mol Med **17**(11-12): 1285-94.

Pease, J. E. and R. Horuk (2009). "Chemokine receptor antagonists: Part 1." Expert Opin Ther Pat **19**(1): 39-58.

Pease, J. E. and R. Horuk (2009). "Chemokine receptor antagonists: part 2." Expert Opin Ther Pat **19**(2): 199-221.

Perugorria, M. J., et al. (2012). "Histone methyltransferase ASH1 orchestrates fibrogenic gene transcription during myofibroblast transdifferentiation." Hepatology **56**(3): 1129-39.

Phillips, M., et al. (2006). "Antioxidants versus corticosteroids in the treatment of severe alcoholic hepatitis--a randomised clinical trial." J Hepatol **44**(4): 784-90.

Pinzani, M. (1999). "Liver fibrosis." Springer Semin Immunopathol **21**(4): 475-90.

Pinzani, M. (2002). "PDGF and signal transduction in hepatic stellate cells." Front Biosci **7**: d1720-6.

Pradere, J. P., et al. (2013). "Hepatic macrophages but not dendritic cells contribute to liver fibrosis by promoting the survival of activated hepatic stellate cells in mice." Hepatology **58**(4): 1461-73.

Purohit, V. and D. A. Brenner (2006). "Mechanisms of alcohol-induced hepatic fibrosis: a summary of the Ron Thurman Symposium." Hepatology **43**(4): 872-8.

Qin, X. and B. Gao (2006). "The complement system in liver diseases." Cell Mol Immunol **3**(5): 333-40.

Rahman, A. H. and C. Aloman (2013). "Dendritic cells and liver fibrosis." Biochim Biophys Acta **1832**(7): 998-1004.

Ramachandran, P. and J. P. Iredale (2012). "Macrophages: central regulators of hepatic fibrogenesis and fibrosis resolution." J Hepatol **56**(6): 1417-9.

Ramaiah, S. K. and H. Jaeschke (2007). "Role of neutrophils in the pathogenesis of acute inflammatory liver injury." Toxicol Pathol **35**(6): 757-66.

Rao, R. (2009). "Endotoxemia and gut barrier dysfunction in alcoholic liver disease." Hepatology **50**(2): 638-44.

Rius, B., et al. (2012). "Resolution of inflammation in obesity-induced liver disease." Front Immunol **3**: 257.

Rossi, D. L., et al. (1997). "Identification through bioinformatics of two new macrophage proinflammatory human chemokines: MIP-3alpha and MIP-3beta." J Immunol **158**(3): 1033-6.

Rot, A. and U. H. von Andrian (2004). "Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells." Annu Rev Immunol **22**: 891-928.

Sahin, H. and H. E. Wasmuth (2012). "Chemokines in tissue fibrosis." Biochim Biophys Acta **1832**(7): 1041-8.

Saitoh, T., et al. (2003). "TWEAK induces NF-kappaB2 p100 processing and long lasting NF-kappaB activation." J Biol Chem **278**(38): 36005-12.

Sancho-Bru, P., et al. (2012). "Liver progenitor cell markers correlate with liver damage and predict short-term mortality in patients with alcoholic hepatitis." Hepatology **55**(6): 1931-41.

Sander, L. E., et al. (2011). "Detection of prokaryotic mRNA signifies microbial viability and promotes immunity." Nature **474**(7351): 385-9.

Santodomingo-Garzon, T., et al. (2009). "Natural killer T cells regulate the homing of chemokine CXC receptor 3-positive regulatory T cells to the liver in mice." Hepatology **49**(4): 1267-76.

Saxena, N. K., et al. (2007). "Concomitant activation of the JAK/STAT, PI3K/AKT, and ERK signaling is involved in leptin-mediated promotion of invasion and migration of hepatocellular carcinoma cells." Cancer Res **67**(6): 2497-507.

Sayana, S. and H. Khanlou (2009). "Maraviroc: a new CCR5 antagonist." Expert Rev Anti Infect Ther **7**(1): 9-19.

Schutyser, E., et al. (2003). "The CC chemokine CCL20 and its receptor CCR6." Cytokine Growth Factor Rev **14**(5): 409-26.

Schwabe, R. F., et al. (2003). "Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration." Am J Physiol Gastrointest Liver Physiol **285**(5): G949-58.

Schwabe, R. F., et al. (2006). "Toll-like receptor signaling in the liver." Gastroenterology **130**(6): 1886-900.

Seki, E. and D. A. Brenner (2008). "Toll-like receptors and adaptor molecules in liver disease: update." Hepatology **48**(1): 322-35.

Seki, E., et al. (2009). "CCR1 and CCR5 promote hepatic fibrosis in mice." J Clin Invest **119**(7): 1858-70.

Seki, E., et al. (2009). "CCR2 promotes hepatic fibrosis in mice." Hepatology **50**(1): 185-97.

Seki, E., et al. (2007). "TLR4 enhances TGF-beta signaling and hepatic fibrosis." Nat Med **13**(11): 1324-32.

Seki, E. and B. Schnabl (2011). "Role of innate immunity and the microbiota in liver fibrosis: crosstalk between the liver and gut." J Physiol **590**(Pt 3): 447-58.

Seki, S., et al. (2011). "Antitumor immunity produced by the liver Kupffer cells, NK cells, NKT cells, and CD8 CD122 T cells." Clin Dev Immunol **2011**: 868345.

Setshedi, M., et al. (2010). "Acetaldehyde adducts in alcoholic liver disease." Oxid Med Cell Longev **3**(3): 178-85.

Shafiei, M. S. and D. C. Rockey (2006). "The role of integrin-linked kinase in liver wound healing." J Biol Chem **281**(34): 24863-72.

Shen, Z., et al. (2009). "Role of SIRT1 in regulation of LPS- or two ethanol metabolites-induced TNF-alpha production in cultured macrophage cell lines." Am J Physiol Gastrointest Liver Physiol **296**(5): G1047-53.

Shi, Z., et al. (1997). "Strain-specific differences in mouse hepatic wound healing are mediated by divergent T helper cytokine responses." Proc Natl Acad Sci U S A **94**(20): 10663-8.

Sica, A., et al. (2013). "Macrophage plasticity and polarization in liver homeostasis and pathology." Hepatology.

Sica, A. and A. Mantovani (2012). "Macrophage plasticity and polarization: in vivo veritas." J Clin Invest **122**(3): 787-95.

Singal, A. K., et al. (2011). "Impact of hepatitis C virus infection on the course and outcome of patients with acute alcoholic hepatitis." Eur J Gastroenterol Hepatol **23**(3): 204-9.

Song, K., et al. (2001). "TH1 cytokine response of CD57+ T-cell subsets in healthy controls and patients with alcoholic liver disease." Alcohol **24**(3): 155-67.

Song, K., et al. (2002). "Chronic ethanol consumption by mice results in activated splenic T cells." J Leukoc Biol **72**(6): 1109-16.

Srikureja, W., et al. (2005). "MELD score is a better prognostic model than Child-Turcotte-Pugh score or Discriminant Function score in patients with alcoholic hepatitis." J Hepatol **42**(5): 700-6.

Svegliati-Baroni, G., et al. (2005). "Early response of alpha2(I) collagen to acetaldehyde in human hepatic stellate cells is TGF-beta independent." Hepatology **42**(2): 343-52.

Szabo, G. and S. Bala (2013). "MicroRNAs in liver disease." Nat Rev Gastroenterol Hepatol **10**(9): 542-52.

Szabo, G., et al. (2006). "Pattern recognition receptors: a contemporary view on liver diseases." Hepatology **44**(2): 287-98.

Szabo, G., et al. (2012). "Innate immunity and alcoholic liver disease." Dig Dis **30 Suppl 1**: 55-60.

Tagami, A., et al. (2003). "Fas-mediated apoptosis in acute alcoholic hepatitis." Hepatogastroenterology **50**(50): 443-8.

Taieb, J., et al. (1998). "Raised plasma soluble Fas and Fas-ligand in alcoholic liver disease." Lancet **351**(9120): 1930-1.

Takahashi, T., et al. (1993). "Induction of cytochrome P-4502E1 in the human liver by ethanol is caused by a corresponding increase in encoding messenger RNA." Hepatology **17**(2): 236-45.

Tanabe, K., et al. (2003). "Fibroblast growth factor-inducible-14 is induced in axotomized neurons and promotes neurite outgrowth." J Neurosci **23**(29): 9675-86.

Theise, N. D., et al. (1999). "The canals of Hering and hepatic stem cells in humans." Hepatology **30**(6): 1425-33.

Thiele, G. M., et al. (2010). "Autoimmune hepatitis induced by syngeneic liver cytosolic proteins biotransformed by alcohol metabolites." Alcohol Clin Exp Res **34**(12): 2126-36.

Thurman, R. G. (1998). "II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin." Am J Physiol **275**(4 Pt 1): G605-11.

Thurman, R. G., et al. (1998). "The role of gut-derived bacterial toxins and free radicals in alcohol-induced liver injury." J Gastroenterol Hepatol **13 Suppl**: S39-50.

Tilg, H. (2001). "Cytokines and liver diseases." Can J Gastroenterol **15**(10): 661-8.

Tilg, H. and A. M. Diehl (2000). "Cytokines in alcoholic and nonalcoholic steatohepatitis." N Engl J Med **343**(20): 1467-76.

Tilg, H., et al. (2006). "How to modulate inflammatory cytokines in liver diseases." Liver Int **26**(9): 1029-39.

Titos, E., et al. (2011). "Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype." J Immunol **187**(10): 5408-18.

Tome, S. and M. R. Lucey (2004). "Review article: current management of alcoholic liver disease." Aliment Pharmacol Ther **19**(7): 707-14.

Tracey, K. J. and A. Cerami (1993). "Tumor necrosis factor, other cytokines and disease." Annu Rev Cell Biol **9**: 317-43.

Tran, N. L., et al. (2003). "The human Fn14 receptor gene is up-regulated in migrating glioma cells in vitro and overexpressed in advanced glial tumors." Am J Pathol **162**(4): 1313-21.

Tran, N. L., et al. (2006). "Increased fibroblast growth factor-inducible 14 expression levels promote glioma cell invasion via Rac1 and nuclear factor-kappaB and correlate with poor patient outcome." Cancer Res **66**(19): 9535-42.

Tran, N. L., et al. (2005). "The tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-fibroblast growth factor-inducible 14 (Fn14) signaling system regulates glioma cell survival via NFkappaB pathway activation and BCL-XL/BCL-W expression." J Biol Chem **280**(5): 3483-92.

Uesugi, T., et al. (2001). "Toll-like receptor 4 is involved in the mechanism of early alcohol-induced liver injury in mice." Hepatology **34**(1): 101-8.

Uesugi, T., et al. (2002). "Role of lipopolysaccharide-binding protein in early alcohol-induced liver injury in mice." J Immunol **168**(6): 2963-9.

Utans-Schneitz, U., et al. (1998). "A novel rat CC chemokine, identified by targeted differential display, is upregulated in brain inflammation." J Neuroimmunol **92**(1-2): 179-90.

Varona, R., et al. (1998). "Molecular cloning, functional characterization and mRNA expression analysis of the murine chemokine receptor CCR6 and its specific ligand MIP-3alpha." FEBS Lett **440**(1-2): 188-94.

Veldhoen, M., et al. (2006). "TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells." Immunity **24**(2): 179-89.

Vinas, O., et al. (2003). "Human hepatic stellate cells show features of antigen-presenting cells and stimulate lymphocyte proliferation." Hepatology **38**(4): 919-29.

Volpe, E., et al. (2008). "A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses." Nat Immunol **9**(6): 650-7.

Wahl, S. M., et al. (1990). "Macrophage production of TGF-beta and regulation by TGF-beta." Ann N Y Acad Sci **593**: 188-96.

Wan, J., et al. (2013). "M2 Kupffer cells promote M1 Kupffer cell apoptosis: A protective mechanism against alcoholic and nonalcoholic fatty liver disease." Hepatology.

Wang, H. J., et al. (2012). "Inflammation in alcoholic liver disease." Annu Rev Nutr **32**: 343-68.

Wasmuth, H. E., et al. (2009). "Antifibrotic effects of CXCL9 and its receptor CXCR3 in livers of mice and humans." Gastroenterology **137**(1): 309-19, 319 e1-3.

Wasmuth, H. E., et al. (2010). "Chemokines in liver inflammation and fibrosis." Semin Liver Dis **30**(3): 215-25.

Werner, S. and R. Grose (2003). "Regulation of wound healing by growth factors and cytokines." Physiol Rev **83**(3): 835-70.

Wesch, D., et al. (2006). "Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes." J Immunol **176**(3): 1348-54.

Wheeler, M. D., et al. (2001). "The role of Kupffer cell oxidant production in early ethanol-induced liver disease." Free Radic Biol Med **31**(12): 1544-9.

Wiley, S. R., et al. (2001). "A novel TNF receptor family member binds TWEAK and is implicated in angiogenesis." Immunity **15**(5): 837-46.

Wilson, N. J., et al. (2007). "Development, cytokine profile and function of human interleukin 17-producing helper T cells." Nat Immunol **8**(9): 950-7.

Winkles, J. A. (2008). "The TWEAK-Fn14 cytokine-receptor axis: discovery, biology and therapeutic targeting." Nat Rev Drug Discov **7**(5): 411-25.

Wynn, T. A. and L. Barron (2010). "Macrophages: master regulators of inflammation and fibrosis." Semin Liver Dis **30**(3): 245-57.

Yang, M. D., et al. (2012). "Rosmarinic acid and baicalin epigenetically derepress peroxisomal proliferator-activated receptor gamma in hepatic stellate cells for their antifibrotic effect." Hepatology **55**(4): 1271-81.

Yin, M., et al. (2001). "Reduced early alcohol-induced liver injury in CD14-deficient mice." J Immunol **166**(7): 4737-42.

Zakhari, S. (2006). "Overview: how is alcohol metabolized by the body?" Alcohol Res Health **29**(4): 245-54.

Zaldivar, M. M., et al. (2010). "CXC chemokine ligand 4 (Cxcl4) is a platelet-derived mediator of experimental liver fibrosis." Hepatology **51**(4): 1345-53.

Zeng, H., et al. (2006). "Flagellin/TLR5 responses in epithelia reveal intertwined activation of inflammatory and apoptotic pathways." Am J Physiol Gastrointest Liver Physiol **290**(1): G96-G108.

Zhang, H. and G. G. Meadows (2009). "Exogenous IL-15 in combination with IL-15R alpha rescues natural killer cells from apoptosis induced by chronic alcohol consumption." Alcohol Clin Exp Res **33**(3): 419-27.

Zhang, Z., et al. (2011). "Hypercytolytic activity of hepatic natural killer cells correlates with liver injury in chronic hepatitis B patients." Hepatology **53**(1): 73-85.

Zhao, X. J., et al. (2008). "TRIF and IRF-3 binding to the TNF promoter results in macrophage TNF dysregulation and steatosis induced by chronic ethanol." J Immunol **181**(5): 3049-56.

Ziol, M., et al. (2001). "Clinical and biological relevance of hepatocyte apoptosis in alcoholic hepatitis." J Hepatol **34**(2): 254-60.

Zlotnik, A. and O. Yoshie (2000). "Chemokines: a new classification system and their role in immunity." Immunity **12**(2): 121-7.

ANNEX I:

SUPPLEMENTARY MATERIAL AND METHODS

1. EXPERIMENTAL ANIMAL MODELS OF LIVER INJURY

All animal procedures were approved by the Ethics Committee of the University of Barcelona and the models performed at Medical Centre of Columbia University were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and by the Columbia University Institutional Animal Care and Use Committee and are in accordance with those set by the National Institutes of Health.

Because the lack of well established animal models of human AH, we performed different animal models of acute, chronic and acute-on-chronic liver injury in mice, trying to reproduce some of the most important features observed in patients with AH such as alcohol binge drinking, fibrosis, and endotoxemia. In our models we used both C57BL/6 and Balb/c mice. The use of Balb/c mice was preferred in the chronic and acute-on-chronic model of liver injury due to the increased predisposition of these mice to develop fibrosis compared to black C57 mice.

1.1 Acute Model of Liver Injury Induced by CCl_4

Male C57BL/6 mice were intraperitoneally administered with carbon tetrachloride (CCl_4 Sigma-Aldrich; diluted 1:5 in oil) or vehicle (oil) at a dose of 1 ml/kg body weight and were sacrificed 72 hours later. Livers were excised and collected.

1.2 Crotaline plus Acetaminophen-Induced Model of Liver Injury

Female C57BL/6 mice received crotaline or vehicle intraperitoneal injections at a dose of 50 mg/kg every two weeks for a total of 2 injections. Two weeks after the last crotaline administration, mice were fasted for 8 hours with free access to water and then intraperitoneally injected with acetaminophen or vehicle at a dose of 500 mg/kg. Animals were sacrificed 48 hours after the last injection. Livers were excised and collected.

1.3 Chronic Model of Oval Cell Expansion Induced by DDC Diet

Male C57BL/6 mice were fed a 0.1% 3,5-diethoxycarbonyl-1,4-dihydro-collidin (DDC) diet for up to 4 weeks. Control mice were fed a standard rodent chow. Animals were sacrificed at the end of the treatment, liver were excised and collected.

1.4 Chronic Model of Liver Injury Induced by CCl_4

A chronic liver injury model was performed by injecting male Balb/c mice with carbon tetrachloride (CCl_4) intraperitoneally (diluted 1:4 in corn oil) at dose of 0.5ml/kg body weight twice per week for a total of 5 injections. Control mice were given vehicle (corn oil) at the same dose. Mice were sacrificed 48 hours after the last CCl_4 injection. Livers were excised and collected.

1.5 Acute and Acute-on-Chronic Models of Liver Injury Induced by Ethanol and CCl_4 plus Ethanol

For the acute model of ethanol gavage, we used male Balb/c mice. Mice were fasted for 8 hours with free access to water and were gavaged a single dose of 50% ethanol (5g/kg body weight, diluted in water) or water. Animals were sacrificed 8 hours after gavage. In the acute-on-chronic ethanol-induced liver injury model, male Balb/c mice were intraperitoneally administered with CCl_4 (diluted 1:4 in corn oil) or vehicle (corn oil) at a dose of 0.5 ml/kg body weight twice per week for a total of 5 injections. Two days after the last CCl_4 injection, mice were fasted for 8 hours with free access to water, and then they were gavaged a single dose of 50% ethanol (5g/kg body weight diluted in water) or water and sacrificed 8 hours after gavage. Livers were excised and collected.

1.6 Acute and Acute-on-Chronic LPS-induced Models of Liver Injury

Balb/c male mice were injected intravenously with lipopolysaccharide (LPS) 10mg/kg body weight or saline as control and were sacrificed 4 hours after the injection. To mimic the effects of endotoxemia in the context of chronic liver disease we performed a model of acute-on-chronic liver injury. Mice were injected with CCl_4 as described in the chronic model and additionally, two days after the last CCl_4 injection, animals were administrated intravenously LPS 10mg/kg body weight and sacrificed 4 hours later. Livers were excised and collected.

1.7 CCL20 Knockdown *in vivo* Using shRNA in LPS-Induced Liver Injury

The effects of CCL20 *in vivo* were evaluated in male C57BL/6 mice. Mice were injected intraperitoneally with 50µg of control shRNA (scrambled sequence) or with shRNA specific for CCL20 complexed with *in vivo* jet-PEI® reagent in a final volume of 400µl. 24 hours later mice were injected intravenously with LPS or saline at dose 2,5mg/kg body weight and immediately after received a second intraperitoneal injection of 50µg of control shRNA or CCL20 specific shRNA. Mice were sacrificed 24 hours after the last injection. Livers were excised and collected. *In vivo* jet-PEI® transfectant agent is a linear polyethylenimine which mediates efficient nucleic acid delivery when combined to 10% isotonic glucose solution (w/v) and forms small and stable complexes. The concentration of shRNA in the final injection solution does not exceed 0.5 µg/µl and, to avoid precipitation, shRNA was resuspended in sterile double distilled water. The volume of reagent was defined by the N/P ratio following manifacturer's instructions and we used a ratio N/P=7 in our experiments.

1.7.1 Protocol of shRNA Preparation and Delivery

1. Dilute the shRNA using the 10% glucose stock solution (provided) and sterile water to prepare a solution of ½ the injection volume of 5% glucose (200µl). Vortex gently or mix by pipetting up and down
2. Dilute the *in vivo* jet-PEI® reagent using the 10% glucose stock solution and sterile water to prepare a solution of ½ the injection volume of 5% glucose (200µl). Vortex gently and spin down.
3. Add the diluted *in vivo* jet-PEI® to the diluted shRNA, vortex gently and spin down.
4. Incubate for 15 minutes at room temperature. (Complexes are stable for 2 hours at room temperature and up to 24 hours if stored at 4ºC, we always used fresh prepared complexes to avoid precipitation and degradation).
5. Perform the intraperitoneal injection of the 400µl of complexes for each mouse using complexes equilibrated at room temperature.

2. MURINE HEPATIC CELLS ISOLATION

Different hepatic cell populations were isolated from livers of mice with acute-on-chronic (CCl_4 + LPS) liver injury and in the model of CCL20 knockdown in order to assess the cell source of CCL20 in mice subjected to these treatment. We used two different protocols and gradients to obtain different hepatic cells. By retrograde and two-step collagenase-pronase perfusion of livers followed by 17,7% Nycodenz two-layer discontinuous density gradient centrifugation we obtained and FACS sorted: macrophages (F4/80+), neutrophils (Ly6G+), T cells (CD3+), and hepatic stellate cells (HSCs) (Vitamin A+). We also collected hepatocytes after the first slow centrifugation and before doing the gradient. Because we also wanted to investigate the expression of CCL20 in activated HSCs and because some activated stellate cells seem to lose Vitamin A, we also performed another retrograde two-step collagenase-pronase perfusion of livers followed by 9,7% Nycodenz two-layer discontinuous density gradient, which is specific for HSCs.

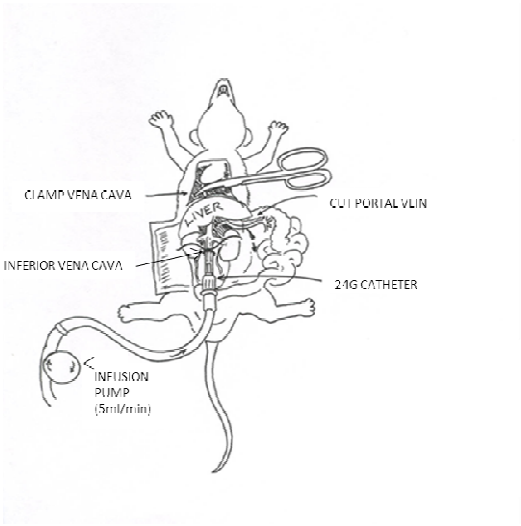


Figure 1. *Retrograde liver perfusion*

Reagents:

- ✓ Collagenase D
- ✓ DNAse I (stock 2 mg/ml)
- ✓ Protease Type XIV
- ✓ Nycodenz
- ✓ SC-1 EGTA SOLUTION
- ✓ SC-2 ENZYME SOLUTION
- ✓ GBSS-A (homemade)
- ✓ GBSS-B (homemade or commercial)

Solutions:

All the solutions are resuspended in double distilled water, and pH adjusted (pH: 7.35~7.4)

	EGTA solution SC-1	**Collagenase solution SC-2**
NaCl	8000mg/L	8000mg/L
Kcl	400mg/L	400mg/L
NaH2PO4. H2O	88.17mg/L	88.17mg/L
Na2HPO4	120.45mg/L	120.45mg/L
HEPES	2380mg/L	2380mg/L
NaHCO3	350mg/L	350mg/L
EGTA	190mg/L	(-)
Glucose	900mg/L	(-)
CaCl2. 2H2O	(-)	560mg/L add slowly after stirring 30min

	GBSS/A SC-3	**GBSS/B SC-4**
NaCl	(-)	8000mg/L
Kcl	370mg/L	370mg/L
MgCl2. 6H2O	210mg/L	210mg/L
MgSO4. 7H2O	70mg/L	70mg/L
Na2HPO4.	59.6mg/L	59.6mg/L
KH2PO4	30mg/L	30mg/L
Glucose	991mg/L	991mg/L
NaHCO3	227mg/L	227mg/L
CaCl2. 2H2O	225mg/L	225mg/L

2.1 Hepatic Cells Isolation Using 17,7% Nycodenz Gradient Solutions (for 3 mice):

A. EGTA SOLUTION- Prepare 100 ml of SC-1; filter and use sterile.

B. PRONASE SOLUTION- Add 40mg of Protease XIV to sterile filtered 100ml of SC-2 Enzyme solution.

C. COLLAGENASE SOLUTION- Add 50mg of Collagenase D to sterile filtered 120ml of SC-2 Enzyme solution (use 0,09 U/ml, the activity of the enzyme dependent on lot; may need to be adjusted for mice with chronic CCl4 or BDL due to fibrotic environment).

D. PRONASE/COLLAGENASE SOLUTION- Add 50mg of Protease XIV and 40mg of Collagenase D in 100 ml of sterile filtered of SC-2, and prior to add perfused-minced liver add 500ul of DNAse I (stock 2mg/ml)

E. NYCODENZ- Prepare 15ml for each set of gradient using 8g of Nycodenz powder in 15ml of homemade GBSS-A to obtain Nycodenz at concentration of 53,3%. Need 15 ml for each mouse or pool of mouse. Agitate on shaker or rocker to dissolve; adjust volume to 15ml filter sterile after dissolving completely and keep on ice before doing the gradient.

For all solutions, add enzyme into sterile bottle and filter respective solutions into the bottle using 0.2µm bottle top filter and mix thoroughly. Incubate all solutions in 40º-42ºC waterbath.

Retrogade Liver Perfusion:

1. Anesthetize mouse and open peritoneal cavity. Move organs to expose inferior vena cava (IVC) and hepatic portal vein.
2. IVC canulation using 24G catheter. Remove needle and ensure backflow of blood. Attach end of the line to catheter. Cut portal vein to allow blood to drain.
3. Cut a small piece of RNA from right lobe and flash freeze for RNA isolation and/or protein extraction.
4. Open chest cavity and clamp down supra-hepatic IVC. Perfuse with EGTA solution (A) until liver get pale, 1-2 minutes at speed of 5ml/min.
5. Perfuse Pronase solution (B) for 5 minutes at speed of 5ml/min.
6. Perfuse Collagenase Solution (C) for 7 minutes at speed of 5ml/min.
7. Remove liver and mince well on 100ml petri dish with small amount of Pronase plus Collagenase Solution (D). Add into flask with Pronase plus Collagenase Solution (D) and 1% DNaseI and stir at 37ºC on hotplate for 25 minutes.
8. Filter the digested liver through cell strainer (70µm) into a 50ml Falcon tube.
9. Centrifuge at 50xg for 3 minutes at 4°C.

10. Transfer supernatant to new 50ml Falcon, and keep pellet on ice (this pellet contains hepatocytes, centrifuge at 1500 rpm for 5 minutes and freeze at -80ºC for RNA and/or protein extraction).

11. Spin supernatant at 580xg for 5 minutes at 4°C.

12. Aspirate supernatant up to 5ml. Add 5ml of GBSSB and 120ul DNAseI (stock 2mg/ml) and resuspend. Fill volume up to 50ml of GBSSB.

13. Spin at 580xg for 10 minutes at 4°C. While spinning, filter Nycodenz solution through syringe filter. Keep on ice.

14. Aspirate up to 5ml of supernatant. Add 5ml GBSSB and 120ul of DNAseI (stock 2mg/ml). Resuspend and thoroughly mix. Fill up volume to 30ml of GBSS-B.

15. Add 15ml of Nycodenz solution and mix well (final volume=45ml, Nycodenz diluted 1:3, Nycodenz starting concentration 53,3%, dilued 1:3 for a final concentration Nycodenz of 17,7%).

16. Transfer 11ml of solution into each of 4 clear 15ml Falcon tubes.

17. Carefully overlay 1ml of GBSSB onto the solution using a syringe.

18. Centrifuge at 1380xg for 15 minutes at 4°C with no brake.

19. Under the clear layer of GBSSB solution, you can see white-brownish layer which contains HSCs, Kupffer cells, T cells, neutrophils, endothelial cells and small hepatocytes. Collect this layer and transfer into new 50ml tube.

20. Fill up with GBSSB to 50ml to wash and centrifuge at 50xg for 2min at 4°C to pellet out hepatocytes.

21. Collect supernatant and transfer to fresh tube; centrifuge at 700xg for 5 minutes at 4°C.

22. Discard supernatant, being careful when aspirating not to disturb pellet and resuspend pellet in appropriate volume of FACS buffer (1-3ml for large visible pellets), count cells and prepare cells in FACS tubes.

2.2 Hepatic Cells Isolation Using 9,7% Nycodenz Gradient Solutions (for 3 mice):

A. EGTA SOLUTION- Prepare 100 ml of SC-1; filter and use sterile.

B. PRONASE SOLUTION- Add 40mg of Protease XIV to sterile filtered 100ml of SC-2 Enzyme solution.

C. COLLAGENASE SOLUTION- Add 50mg of Collagenase D to sterile filtered 120ml of SC-2 Enzyme solution (use 0,09 U/ml, the activity of the enzyme dependent on lot; adjust for mice with chronic CCl4 due to fibrotic environment).

D. PRONASE/COLLAGENASE SOLUTION- Add 50mg of Protease XIV and 40mg of Collagenase D in 100ml of sterile filtered of SC-2, and prior to add perfused-minced liver add 500ul of DNAse I (stock 2mg/ml)

E. NYCODENZ- Prepare 17ml for each set of gradient using 4.94g of Nycodenz in 17ml of GBSSA to obtain Nycodenz at concentration of 29,05%. Need 15 ml for each mouse or pool of mouse, agitate on a shaker or rocker to dissolve and keep on ice before doing the gradient.

For all solutions, add enzyme into sterile bottle and filter respective solutions into the bottle using 0.2µm bottle top filter and mix thoroughly. Incubate all solutions in 40º-42ºC waterbath.

Retrogade Liver Perfusion:

1. Anesthetize mouse and open peritoneal cavity. Move organs to expose IVC and hepatic portal vein.
2. Cannulate the IVC using 24G catheter. Remove needle and ensure backflow of blood. Attach end of the line to catheter. Cut portal vein to allow blood to drain.
3. Cut a small piece of RNA from right lobe and flash freeze for RNA isolation and/or protein extraction.
4. Open chest cavity and clamp down suprahepatic IVC.
5. Perfuse with EGTA solution (A) only until liver gets pale (about 1-2 minutes).
6. Perfuse Pronase solution (B) for 5 minutes at speed of 5ml/min.
7. Perfuse Collagenase Solution (C) for 7 minutes at speed of 5ml/min.
8. Remove liver and mince well on 100ml petri dish with small amount of Solution D. Add into flask with Solution D and 1% DNaseI and stir at 37ºC on hotplate for 25 minutes.
9. Filter digested liver through cell strainer (70µm) into a 50ml Falcon tube.
10. Spin supernatant at 580xg for 10 minutes at 4C.
11. Aspirate supernatant up to 5ml. Add 5ml of GBSSB and 120ul DNAseI and resuspend. Fill volume up to 50ml of GBSSB.
12. Spin at 580xg for 10 minutes at 4ºC. While spinning, filter Nycodenz solution through syringe filter. Keep on ice.
13. Aspirate up to 5ml of supernatant. Add 5ml GBSSB and 120ul of DNAseI. Resuspend and thoroughly mix. Fill up volume to 32ml of GBSSB.
14. Add 16ml of Nycodenz solution and mix well to obtain a 9,7% of final Nycodenz concentration.
15. Transfer 11.5-12ml of solution into each of 4 clear 15ml Falcon tubes.
16. Carefully overlay 1ml of GBSSB onto the solution.
17. Centrifuge at 1380xg for 17 minutes at 4ºC with no brake.
18. Under the clear layer of GBSSB solution, you can see white layer which contains HSCs. Collect this layer and transfer into new 50ml tube.
19. Fill up with GBSSB to 50ml to wash and centrifuge at 580xg for 10min at 4ºC.

20. Discard supernatant, be careful when aspirating not to disturb pellet and resuspend pellet in appropriate volume of DMEM to count cells usually (1-3ml for large visible pellets) or freeze pellet at -80ºC for RNA and/or protein extraction.

ANNEX II: EDITORIAL ARTICLE

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

RANTES antagonism: A promising approach to treat chronic liver diseases

Silvia Affò, Ramón Bataller*

Liver Unit, Hospital Clínic, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Centro de Investigación Biomèdica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Barcelona Catalonia, Spain

COMMENTARY ON:

Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. Marie-Luise Berres, Rory R. Koenen, Anna Rueland, Mirko Moreno Zaldivar, Daniel Heinrichs, Hacer Sahin, Petra Schmitz, Konrad L. Streetz, Thomas Berg, Nikolaus Gassler, Ralf Weiskirchen, Amanda Proudfoot, Christian Weber, Christian Trautwein, and Hermann E. Wasmuth. The Journal of Clinical Investigation 2010;120(11):4129–4140. Copyright 2010. Abstract reprinted with permission of the American Society for Clinical Investigation.

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2964968/

Abstract: *Activation of hepatic stellate cells in response to chronic inflammation represents a crucial step in the development of liver fibrosis. However, the molecules involved in the interaction between immune cells and stellate cells remain obscure. Herein, we identify the chemokine CCL5 (also known as RANTES), which is induced in murine and human liver after injury, as a central mediator of this interaction. First, we showed in patients with liver fibrosis that CCL5 haplotypes and intrahepatic CCL5 mRNA expression were associated with severe liver fibrosis. Consistent with this, we detected Ccl5 mRNA and CCL5 protein in 2 mouse models of liver fibrosis, induced by either injection of carbon tetrachloride (CCl_4) or feeding on a methionine and choline–deficient (MCD) diet. In these models, $Ccl5^{-/-}$ mice exhibited decreased hepatic fibrosis, with reduced stellate cell activation and immune cell infiltration. Transplantation of Ccl5-deficient bone marrow into WT recipients attenuated liver fibrosis, identifying infiltrating hematopoietic cells as the main source of Ccl5. We then showed that treatment with the CCL5 receptor antagonist Met-CCL5 inhibited cultured stellate cell migration, proliferation, and chemokine and collagen secretion. Importantly, in vivo administration of Met-CCL5 greatly ameliorated liver fibrosis in mice and was able to accelerate fibrosis regression. Our results define a successful therapeutic approach to reduce experimental liver fibrosis by antagonizing Ccl5 receptors.*

Keywords: RANTES, Chemokines; Liver fibrosis; Hepatic stellate cells; Inflammation.

Received 4 March 2011; received in revised form 8 April 2011; accepted 11 April 2011

* Corresponding author. Address: Liver Unit, Hospital Clinic, Villarroel 170, 08015 Barcelona Catalonia, Spain. Tel.: +34 932275400x3371; fax: +34 932275522.

E-mail address: bataller@clinic.ub.es (R. Bataller).

Abbreviations: HSC, hepatic stellate cells; RANTES, regulated upon activation normal T-cell expressed and secreted; CCl_4, carbon tetrachloride; HCV, hepatitis C virus.

Most types of chronic liver disease are characterized by different degrees of hepatocellular damage, inflammatory cell infiltrate in the hepatic parenchyma, and tissue remodeling, ultimately resulting in progressive fibrosis and cirrhosis. Following repeated liver injury, a complex interplay between damaged hepatocytes, inflammatory cells, and non-parenchymal cells occurs. Infiltrating inflammatory cells at the sites of liver injury are directed to remove apoptotic cells but in addition they secrete a number of chemokines that stimulate resident cells such as hepatic stellate cells (HSCs). The resulting activated HSCs proliferate and accumulate in the injured liver, secreting large amounts of extracellular matrix proteins. Therefore, chemokines are currently considered key drivers of liver fibrogenesis and potential targets for therapy [1–3]. Chemokines are chemotactic cytokines that regulate the movement of circulating leukocytes by binding to their specific seven-transmembrane domain G-protein-coupled receptors [4]. According to the presence and position of a conserved amino-proximal cysteine-containing motif, they are classified into four subfamilies: CC, CXC, CX3C, and C chemokines [3]. CC chemokines are the largest family and are defined by the location of the first two cysteine residues in the sequence, which are adjacent. This group is known also as β-chemokines or 17q chemokine family, due to a gene cluster on human chromosome 17q11–q32 [4,5]. CCL5, also known as "regulated upon activation, normal T-cell expressed, and secreted" (RANTES), is a small CC chemokine that has powerful chemoattracting properties toward T cells, dendritic cells, eosinophils, NK cells, mast cells, and basophils. CCL5 is produced by different cell types including T cells, platelets, macrophages, endothelial cells, and fibroblasts and exerts its actions by binding to three receptors (CCR1, CCR3, and CCR5) [6]. A growing body of evidence indicates that RANTES is involved in a variety of inflammatory conditions including atherosclerosis and obesity, which share common pathophysiological pathways with liver diseases [7,8]. Pharmaceutical companies have recently developed RANTES inhibitors/CCR5 antagonists, which are currently being evaluated in several inflammatory diseases. Moreover, because CCR5 is involved in HIV entry to target cells, CCR5 antagonists have been successfully tested in phase III studies in patients with HIV infection [9].

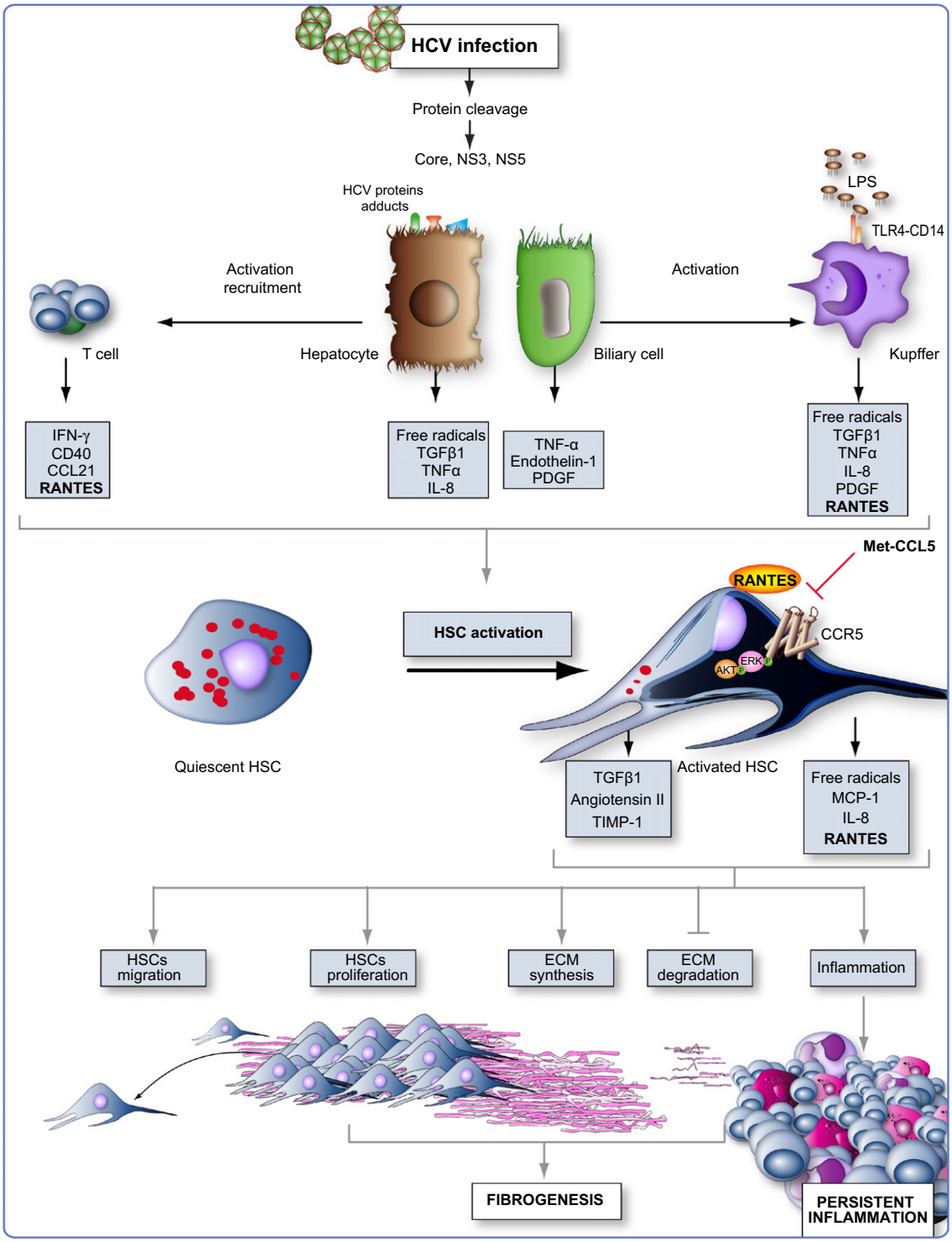


Fig. 1. Implication of RANTES in the pathogenesis of liver fibrosis due to HCV infection. HCV entries hepatocytes and is cleaved into different proteins with biological actions (core, NS3, NS5, etc.). Some of these proteins are expressed in the membrane of hepatocytes together with MHC-I and MHC-II molecules. Moreover, infected hepatocytes secrete inflammatory cytokines. Both actions activate and recruit T-lymphocytes that secrete mediators including RANTES. Neighboring biliary cells and non-parenchymal cells (hepatic stellate cells – HSC and Kupffer cells) become activated and secrete free radicals and fibrogenic and inflammatory mediators. The inflammatory milieu activates resident HSC into myofibroblastic cells. These latter cells express CCR5 and secrete RANTES. Paracrine and autacrine actions of RANTES in HSCs stimulate intracellular signaling pathways leading to increased collagen synthesis, impaired collagen degradation and secretion of further inflammatory mediators. These actions lead to progressive fibrosis and persistent liver inflammation. The use of new RANTES receptor antagonists (e.g. Met-CCL5) could block the pathogenic effects of RANTES and attenuate the progression of liver fibrosis.

Experimental and human evidence indicate that RANTES is implicated in hepatic wound healing response to chronic injury. An initial study demonstrated that RANTES is expressed and secreted by activated HSCs, which are the main collagen-producing cells in the injured liver. In these cells, RANTES induces migration, proliferation, and fibrogenic properties [10]. Moreover, studies in experimental and human liver fibrosis convincingly showed that the CCL5/CCR5 axis is an important system in the hepatic would healing response. This axis is over-expressed in different models of liver fibrosis and in patients with chronic liver diseases such as chronic HCV infection (Fig. 1). Importantly, deletion of CCR5 markedly attenuated liver fibrosis in mice [11]. The underlying mechanisms include modulation of infiltration of T lymphocytes and bone-marrow derived cells and reduced HSCs activation. Finally, recent data suggest that RANTES could be involved in the chemotaxis of progenitor cells during hepatic fibrogenesis and tissue repair [12].

In the paper by Berres *et al.* [6], the authors expanded these previous data by performing a multi-approach study demonstrating that RANTES is a major driver of inflammation and fibrosis in chronic liver injury. First, a genetic analysis indicated that RANTES gene variations influence the degree of liver fibrosis in patients with chronic hepatitis C. The haplotype CCL5_H3 was found more prevalent in patients with advanced fibrosis compared with those with mild fibrosis. Second, RANTES mRNA and protein expression were up-regulated in two experimental models of liver fibrosis as well as in patients with advanced HCV-induced fibrosis. Third, they demonstrated that genetic ablation of RANTES results in attenuated liver fibrosis in mice subjected to two experimental models of liver fibrosis. Absence of RANTES was associated with reduced HSCs activation and immune cell infiltration in the injured liver. By studying bone marrow chimeric mice, they provide evidence that immune cells are the main source of RANTES in liver fibrogenesis, while resident HSCs are probably a target cell type for this chemokine. Finally, the authors tested a recently developed RANTES receptor antagonist (Met-CCL5) *in vitro* and *in vivo*. Met-CCL5 inhibited the proliferation and migration of cultured HSCs as well as chemokine secretion and collagen synthesis. Furthermore, RANTES inhibition attenuated the progression of liver fibrosis in mice treated with CCl_4 and was able to accelerate fibrosis regression after cessation of liver injury.

The study by Berres *et al.* provides convincing pre-clinical evidence that RANTES inhibition is a promising approach to treat chronic liver diseases. However, there are several issues that deserve further attention. The effect of RANTES inhibition on regression of liver fibrosis was only mild and additional studies using different experimental models are needed. Also, the expression of RANTES and its receptors in alcoholic liver disease, which is mainly driven by polymorphonuclear cells, should be explored. Because the authors propose that RANTES is involved in disease progression in chronic hepatitis C, it seems pertinent to explore whether RANTES inhibition modulates HCV cell cycle, replication, and pathogenic effects. For this purpose, *in vitro* replicon systems and transgenic mice are available experimental tools.

Additional studies are also required to better delineate the role of RANTES in liver fibrogenesis and its potential as a target for therapy in humans. Translational studies in different degrees and types of chronic liver diseases should identify the specific cell origin of RANTES. The pathogenic effects of RANTES in mediating hepatocellular injury, endothelial dysfunction, immune disturbances, and collagen synthesis are largely unknown and deserve further investigation. Importantly, future studies should investigate the involvement of CCL5/CCR5 axis in liver regeneration and cancer development. And finally, carefully-designed experimental studies should explore the potential side effects of continuous inhibition in RANTES and/or its receptors in animals with chronic liver injury. In this line, a recent report [13] indicates that lack of CCR5 promotes murine fulminant liver failure by preventing the apoptosis of activated CD1d-restricted NKT cells. This study suggests that prolonged manipulation of chemokine receptors may result in tissue damage instead of resolution of inflammation. Another potential side effect of prolonged RANTES inhibition is inducing immunosuppression. This is particularly important in patients with liver cirrhosis, acute-on-chronic liver disease, and patients with alcoholic liver disease, who are prone to develop severe bacterial infections due to impaired immune defense.

Although chemokines including RANTES are currently considered an appealing family of molecular targets to develop antifibrotic therapies, all these biological and clinical parameters should be carefully considered before testing this type of drugs in patients with chronic liver diseases.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

References

[1] Holt AP, Tacke Salmon, Buckley CD, Adams DH. Immune interactions in hepatic fibrosis. Clin Liver Dis 2008;12:861–882, [x].
[2] Wasmuth HE, Tacke F, Trautwein C. Chemokines in liver inflammation and fibrosis. Semin Liver Dis 2010;30:215–225.
[3] Morales-Ibanez O, Bataller R. Platelet-derived chemokines:New targets to treat liver fibrosis. J Hepatol 2010;154 (3):581–583.
[4] Charo IF, Ransohoff RM. The many roles of chemokine receptors in inflammation. N Engl J Med 2006;354:610–621.
[5] Bonecchi R, Galliera E, Borroni EM, Corsi MM, Locati M, Mantovani A. Chemokines and chemokine receptors: an overview. Front Biosci 2009;14:540–551.
[6] Berres ML, Koenen RR, Rueland A. Antagonism of the chemokine Ccl5 ameliorates experimental liver fibrosis in mice. J Clin Invest 2010;120 (11):4129–4140.
[7] Koenen RR et al. Disrupting functional interactions between platelet chemokines inhibits atherosclerosis in hyperlidemic mice. Nat Med 2009;15 (1):97–103.
[8] Wu H et al. T-cell accumulation and regulated on activation, normal T-cell expressed and secreted upregulation in adipose tissue in obesity. Circulation 2007;115 (8):1029–1038.
[9] Kuhmann SE, Hartley O. Targeting chemokine receptors in HIV: a status report. Annu Rev Pharmacol Toxicol 2008;48:425–461.
[10] Schwabe R, Bataller R, Brenner DA. Human hepatic stellate cells express CCR5 and RANTES to induce proliferatin and migration. Am J Physiol Gastrointest Liver Physiol 2003;285 (5):G949–G958.
[11] Seki E et al. CCR1 and CCR5 promote hepatic fibrosis in mice. J Clin Invest 2009;119 (7):1858–1870.
[12] Ruddel RG, Knight B, Tirnitz-Parker JE, et al. Lymphotoxin-beta receptor signaling regulates hepatic stellate cell function and wound healing in a murine model of chronic liver injury. Hepatology 2009;49:227–239.
[13] Ajuebor MN, Aspinall AI, Zhou F, et al. Lack of chemokine receptor CCR5 promotes murine fulminant liver failure by preventing the apoptosis of activated CD1d-restricted NKT cells. J Immunol 2005;174:8027–8037.

ACKNOWLEDGEMENTS

I would like to thank all the people who have accompanied me during this professional and personal training process.

A special thank you to my thesis directors for welcoming me in the lab family and for making me feel at home until the end of this journey.

I feel so lucky and proud to have met not only amazing lab mates but also special friends during these years; thank you guys for making every day funny or gnecco, and to share happiness and frustration.

Thank you to my husband, my family and my friends for always being there, helping me and always supporting me.

Thank you all for making this thesis so special!