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« Role of RIPK1 in the survival and death of hepatocytes : its involvement in murine hepatitis models»

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LIST OF ABBREVIATIONS

ACD Accidental cell death

ALD Alcoholic liver disease

ALF Acute liver failure

APAF1 Apoptotic protease activating factor 1

APAP N-acetyl-p-aminophenol

APCs Antigen-presenting cells

ASK1 Apoptosis signal-regulating kinase 1

ASO anti-sense oligonucleotide

BAX BCL2 associated X protein

BCL-XL BCL extra-large

BCL2 B cell lymphoma 2

BID BH3 interacting-domain death

Casp-8 Caspase 8

CC3 Cleaved caspase 3

CCl$_3$ Trichloromethyl radical

CCl4 carbon tetra chloride

CD cluster of differentiation

cDCs Classical dendritic cells

cIAP cellular inhibitor of apoptosis

CMV Cytomegalovirus

ConA Concanavalin A

D-Gal D-Galactosamine

DAI DNA-dependent activator of interferon regulatory factors

DAMPs Damage-associated molecular patterns

DCs Dendritic cells

ETA Etanercept

FADD Fas associated protein with DD

FasL Fas ligand

FGL2 Fibrinogen-like protein 2

FHF Fulminant hepatitis failure

GSH glutathione

GSK3 β Glycogen synthase kinase 3 beta

HBV Hepatitis B virus

HCC Hepatocellular carcinoma

HCV Hepatitis C virus

HE Hepatic encephalopathy

HFD High fat diet

HFHCD High fat high cholesterol diet

HSCs Hepatic stellate cells

HSV Herpes simplex virus

IFN Interferons

IHC Immunohistochemistry

IKK IκB kinase

IRAK1 Interleukin 1 receptor associated kinase 1

IRAK4 Interleukin 1 receptor associated kinase 4

JNK c-Jun N-terminal kinase

KCs Kupffer cells

KD Kinase dead

KO Knock-out

LPC Liver parenchymal cells

LPS Lipopolysaccharide

LSECs Liver sinusoidal endothelial cells

MCD Methionine choline deficient diet

MEKK1 Mitogen activated protein kinase kinase 1

MEKK3 Mitogen activated protein kinase kinase 3

MHC-I major histocompatibility complex class I

MHV3 MHV type 3

MKK4 Mitogen-activated-protein-kinase kinase 4

MLK3 Mixed lineage protein kinase 3

MLKL Mixed lineage kinase domain like pseudokinase

MOMP Mitochondrial outer membrane permeabilization

MyD88 Myeloid differentiation primary response 88

NAFLD Non-alcoholic fatty liver disease

NAPQI N-acetyl-p-benzoquinone

NASH Non-alcoholic steatohepatitis

NCCD Nomenclature Committee of Cell Death

Nec-1 Necrostatin-1

NEMO NF-κB essential modulator

NETs Neutrophils extracellular traps

NIAAA National institute on alcohol abuse and alcoholism

NK (Natural Killer)

NKT Natural killer T

NLRs Nod like receptors

NPC Non-parenchymal cells

OxLDL Oxidized low-density lipoproteins

p-MLKL Phosphorylated Mixed lineage kinase domain like pseudokinase

PAMPs Pathogen associated molecular patterns

pDCs Plasmacytoid dendritic

PHH Primary human hepatocytes

PI Propidium iodide

PKC Protein kinase C

PMH Primary mouse hepatocytes

Poly I:C Polyinosinic:polycytidylic acid

PRRs Pattern recognition receptors

RCD Regulated cell death

RDA Ripk1-dependent apoptosis

RHIM RIP homology interaction motif

RIA Ripk1-Independent Apoptosis

RIPK1 Receptor interacting protein kinase 1

RIPK3 Receptor interacting protein kinase 3

ROS reactive oxygen species

Sab SH3 homology associated binding protein

SD Standard diet

SMAC Second mitochondria-derived activator of caspases

TAB2 TAK1 binding protein 2

TAB3 TAK1 binding protein 3

TAK1 Transforming growth factor beta-activated kinase 1

TCR T cell receptor

TGF Transforming growth factor

TIRAP TIR domain containing adapter protein

TLRs toll like receptors

TNF Tumor necrosis factor

TRADD TNF receptor associated DD

TRAF2 TNF receptor-associated factor 2

TRAF5 TNF receptor-associated factor 5

TRAIL Tumor necrosis factor-related apoptosis inducing ligand

TRAM TRIF related adapter molecule

TRIF TIR-domain-containing adapter-inducing interferon Beta

WT Wild type

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I. INTRODUCTION

1   LIVER

1.1   PHYSIOLOGY OF THE LIVER

The liver, the largest internal organ of the body, is located in the abdominal cavity. In human, it is a lobulated organ, which represents 2-3% of the total body weight. It is a vital organ carrying out multiple functions like metabolism, glycogen storage, biosynthesis of proteins and detoxification (1). Its blood supply is unique in a way that it comprises both oxygenated and deoxygenated blood from hepatic artery and portal vein, respectively (Figure 1). Both oxygenated and deoxygenated blood mixes in liver sinusoids. In sinusoids, various toxins are detoxified, and nutrients are absorbed. From sinusoids, blood enters in hepatic vein and ultimately to inferior vena cava. Portal supply exposes the liver to a variety of injurious and non-injurious antigens. On the one hand, liver has to respond in such a way as to efficiently eliminate the injurious antigens and, on the other hand, to tolerate the non-injurious antigens.

Figure 1 General anatomy of the human liver (organsofthebody.com)
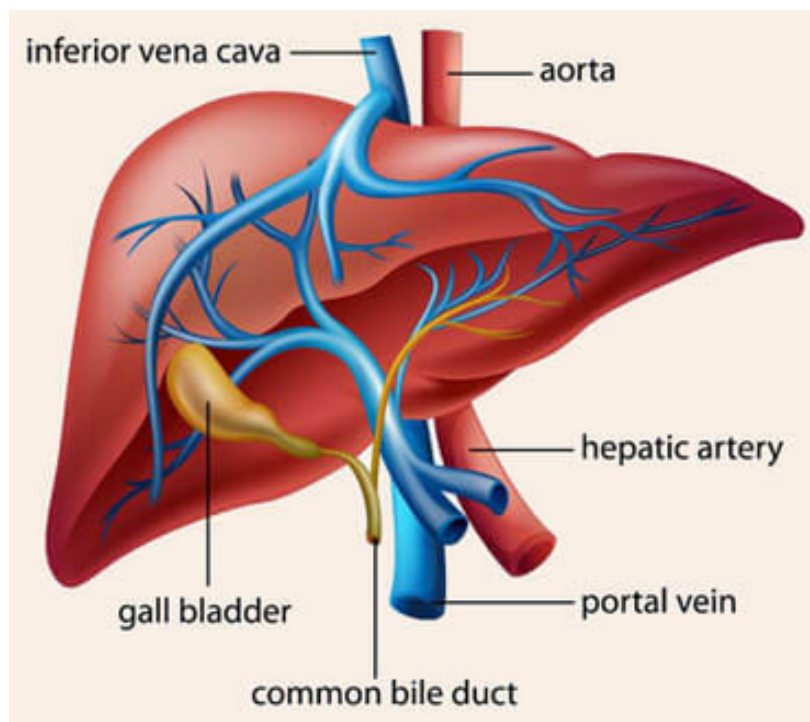
Liver is a unique multi-functional organ. Liver governs various functions including but not limited to metabolism (fats, carbohydrates and proteins, bilirubin), synthesis (various plasma proteins, complement proteins, bile salts), storage (vitamins like ADEK, iron and glycogen) detoxification (drugs, toxins), metabolism and excretion (hormones like steroid hormones) and immunological functions(2). Histologically, the liver is composed of structural and functional units called lobules (figure 2A). Each lobule has a central vein, named due to its unique location in the center of each lobule. On periphery, these lobules have portal triad consisting of portal vein, hepatic artery and bile duct. Hepatocytes are arranged in the form of cords and are separated from liver sinusoids by the space of Disse. Liver sinusoids are lined by fenestrated monolayer of liver sinusoidal endothelial cells (LSECs). Hepatic stellate cells (HSCs), initially named Ito cells, are situated in the space of Disse. Kupffer cells (KCs), which are liver resident macrophages, are located in liver sinusoids. The bile secreted from hepatocytes is transported to bile duct via bile canaliculi. These bile canaliculi are lined by cholangiocytes. The other immune cells like NK (Natural Killer), NKT, B and T cells are present in liver sinusoids (3). Histology of the liver is shown in Figure 2B.

Figure 2: Liver histology. Reproduced with permission (3)
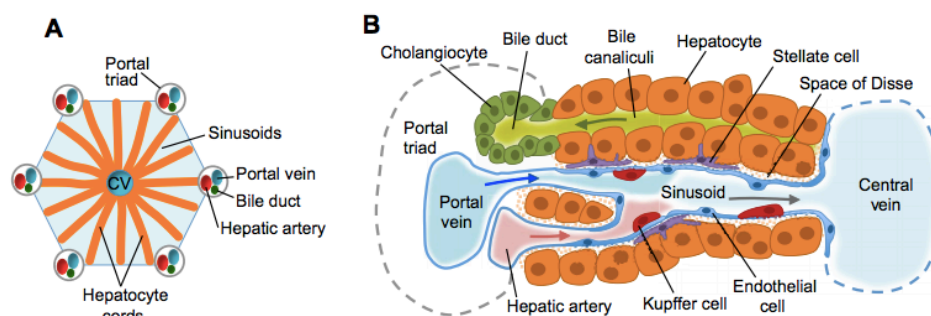
A : Schematic representation of an hepatic lobule (CV, central vein)

B : Cellular organization in the liver

1.2 CELLULAR COMPOSITION

Liver tissue consists of a large variety of cells more or less represented as detailed in figure 3.

Figure 3: Cellular composition of the liver. Reproduced with permission (4)
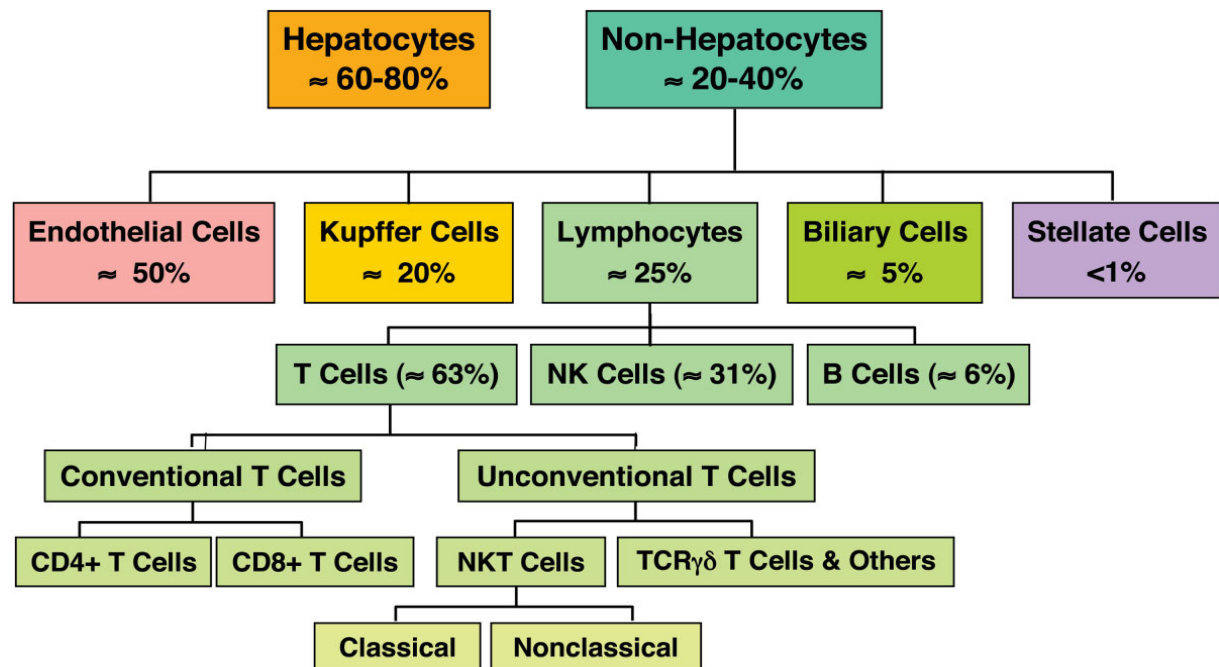
1.2.1 Hepatocytes

Hepatocytes are the main hepatic cells comprising 60-80% of total liver cells and are arranged in the form of cords (4). Hepatocytes play various roles, including metabolism of different nutrients and drugs, synthesis of proteins, detoxification and synthesis of bile. Furthermore, hepatocytes also play key role in liver immunity through the production of various cytokines and chemokines as well as through antigen presentation to other immune effector cells (1).

1.2.2 Cholangiocytes

Cholangiocytes are epithelial cells, which line biliary tree. This biliary tree is further composed of complex network of bile ducts, which transport bile produced by hepatocytes from liver to intestine. The main physiological function of cholangiocytes is to regulate bile composition through coordinated transport of various solutes and water (5).

1.2.3 Kupffer Cells (KCs)

Kupffer cells, also known as liver resident macrophages, are the largest population of tissue macrophages, accounting for 80-90% of total tissue macrophages (6). In liver, KCs represent 20-35% of total non-parenchymal cells (7). These immune cells are found in liver sinusoids and act as first line of defense against invading pathogens. KCs has the ability to produce both pro-inflammatory and anti-inflammatory cytokines, maintaining the balance of inflammation and therefore the immunological tolerance (4). KCs are activated in response to sensing of pathogen associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) by their cognate toll like receptors (TLRs) and Nod like receptors (NLRs) (8). Complement proteins and reactive oxygen species (ROS) can also activate KCs, resulting in pathogen killing through phagocytosis. KCs has also the capacity to kill damaged cells through secretion or presentation of death ligands like tumor necrosis factor alpha (TNF-α), Fas ligand (FasL) and tumor necrosis factor-related apoptosis inducing ligand (TRAIL). In addition, KCs act as antigen-presenting cells (APCs) to coordinate with more specific adaptive immune response (4, 6). Depletion of KCs by pretreatment with clodronate resulted in amelioration of liver damage and decreased cholongio-cellular overgrowth (9). Furthermore, cenicriviroc (C-C motif chemokine receptor 2 antagonist) induced inhibition of monocytes (including KCs) infiltration and improved non-alcoholic steatohepatitis (NASH) (10).

1.2.4 Hepatic dendritic Cells (DCs)

Although rare, the liver contains other APCs, such as the dendritic cells (DCs), specialized bone marrow derived cells. DCs are most commonly localized around central vein and portal tracts (4). They play crucial role in coordination of innate and adaptive immune response. Based on functional disparities, hepatic dendritic cells are categorized into two distinct phenotypes: classical dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs). cDCs are the key cells

in antigen presentation while pDCs produce type I interferons (IFN-α, IFN-β) in response to viral exposure (11). Experimental depletion of DCs resulted in increased liver damage in acetaminophen and NASH mouse models, suggesting its immune regulatory role (12, 13). Based on their lipid contents, DCs can act as immunogenic or tolerogenic, suggesting their regulatory role in NASH. Thus, DCs having high lipids act as immunogenic, while DCs with low lipid contents play an immune tolerogenic role. Therefore, due to lipid rich micro-environment in NASH patients and mice, there is more immunogenic DCs. These immunogenic DCs resulted in increased cytokines levels like TNF-α and IL-4 and IL-6 (14, 15). However, it is not fully elucidated how the lipid contents affect the different mechanism of functioning of the DCs.

1.2.5 Liver Natural Killer cells

In mice, NK cells account for 20-30% of total liver resident lymphocytes, significantly higher as compared to their relative proportion in peripheral blood (5%) (16). They display a surveillance activity, killing infected and tumor cells (17). NK cells have the ability to produce different cytokines like IFN gamma and TNF-α. In experimental viral hepatitis model induced by administration of poly I:C and D-Galactosamine (D-Gal), NK cells act synergistically with KCs to carry out excessive liver damage (18). NK cells express elevated surface TRAIL in response to IFN gamma therapy, which result in the killing of the viral infected cells. Furthermore, NKs can promote apoptosis of HSCs leading to liver fibrogenesis inhibition. In addition to TRAIL, cytotoxic effect of NKs can be due to perforin, FAS or granzyme (17).

1.2.6 Natural killer T (NKT) cells

Liver NKT cells are the type of immune cells, which express both T (TCR T cell receptor) and NK cells markers (17). Unlike T cells, NKT cells recognize lipid antigens, which are exclusively presented by major histocompatibility complex class I (MHC-I) molecules related

cluster of differentiation 1 (CD1 molecules) (19). NKT cells secrete different cytokines as well as act as cytotoxic cells, like NK cells, through presentation of different death ligands and through production of perforin and granzymes. NKT cells are divided into two distinct groups; Type 1 NKT cells also called classical NKT cells, invariant NKT cells or Va 14 iNKT cells, and type 2 NKT cells also called non-classical NKT cells. Classical NKT cells account for 95% of total NKT cells. While classical NKT cells contribute to liver damage in experimental model of autoimmune hepatitis induced by concanavalin A (ConA), non-classical NKT cells play a protective role in the same model (19).

1.2.7   Neutrophils

Neutrophils, bone marrow derived granulocytes, are the most frequent immune cells in human circulation. They are recruited in the liver in response to different cytokines including, but not limited to, TNF-α and IL-1 alpha and beta and chemokines (IL-8 in human and CXCL-1 in mice) secreted during liver damage or infection (20, 21). Their recruitment works as a double-edged sword. On the one hand, it helps to remove dead cells and to contain infection, and on the other hand it can exacerbate liver damage in overwhelming response (20). Neutrophils can contain infection through phagocytosis, production of antimicrobial peptides or through neutrophils extracellular traps (NETs). These NETs are a web of extracellular fibers containing DNA, histones and proteases that can block liver blood flow, resulting in ischemic liver injury (22). Although neutrophil infiltration has been reported in chronic hepatitis, their role is not well characterized (22).

1.2.8   T and B-lymphocytes

Although innate immune response act as a first line of defense to contain invading pathogens, more effective immunity requires the activation of acquired immune response (23). Acquired immunity is typically characterized by specificity and memory for future confrontations.

Acquired immunity is further divided into humoral and cellular immunity carried out through activation of B and T cells, respectively (23). B cells, are less frequent and less characterized cells in liver diseases (24). T cells are further composed of CD4+ and CD8+ cells, based on TCR diversity. CD4+ cells recognize antigen presented through MHC-II molecules, which are specifically presented by professional APCs like dendritic cells. Whereas CD8+ cells recognize antigens presented by MHC-I molecules which are potentially present in all nucleated cells (23). CD4+ cells secrete different pro-inflammatory cytokines like TNF-α and IFN gamma, which coordinate with other immune cells. Effector CD8+ cells, also known as cytotoxic T cells, carry out the killing of infected cells through different death ligands and perforin. CD8+ cells are known to be required for efficient clearance of hepatotropic HBV and HCV viruses (23, 25).

1.2.9   Liver sinusoidal endothelial cells

LSECs, the most abundant liver non-parenchymal cells, lines the liver sinusoids (4). Being the most efficient endocytic cells in the body, LSECs uptakes a wide variety of circulating antigens (26). LSECs also have the ability to present antigens via MHC-I and MHC-II molecules, and coordinate with other immune cells for efficient clearance of pathogens (26).

2   PATHOPHYSIOLOGY OF THE LIVER

The liver is continuously exposed to a variety of antigens. On the one hand, liver should tolerate non-injurious antigen, and on the other hand, it should efficiently respond to contain injurious antigens. Under physiological condition, there is a balance between liver inflammation and its unique ability to regenerate (Figure 4, upper panel). When liver fails to cope with excessive liver damage, it can result in acute liver failure. In prolonged exposure to insults, coupled with compensatory proliferation, liver undergoes chronic hepatitis which can further leads to more serious forms, cirrhosis and hepatocellular carcinoma (HCC) (Figure 4, lower panel). Among

the existing liver pathologies, my research focused on fulminant viral hepatitis and NASH, two conditions that are presented below after introducing a central notion to liver damage. Cell death is a key contributor of liver pathology. Here, we discuss about liver pathologies in NASH and fulminant hepatitis but before that, we will briefly discuss cell death and its involvement in liver diseases.

Figure 4: Role of inflammation in pathophysiology of liver. Reproduced with permission (1)

Liver Homeostasis
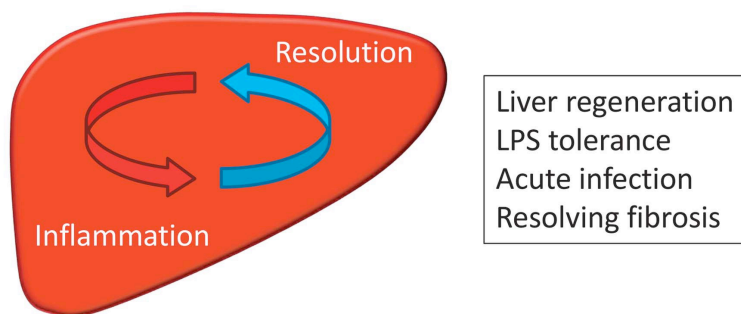
Liver Pathology
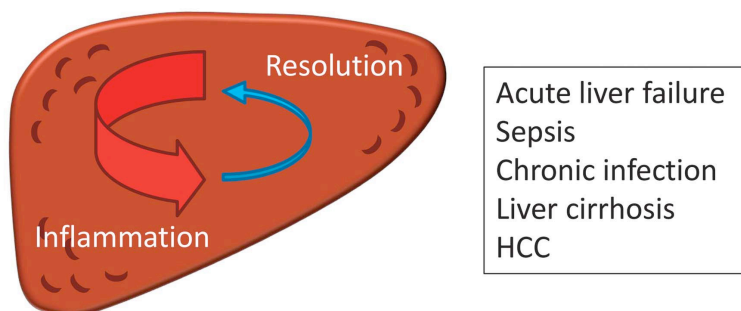
Robinson 2016

2.1   CELL DEATH

Cell death is an integral part of development and homeostasis. In adult, about 10-100 billions cells die daily and are efficiently replaced with normal cells for maintenance of homeostasis. Furthermore, numerous tissues are formed during development, which are removed in later stages of life or depending on the sex. For example, müllerian duct, which leads to development of uterus and fallopian tube, is efficiently removed through cell death in males (27). The

disruption of this delicate balance between cell death and proliferation is detrimental. Excessive death is hallmark of many diseases like fulminant hepatitis and alcoholic liver disease, while resistance to cell death can lead to cancer (28).

Similarly, cell death is a key process in the development and progression of liver diseases. Different complex processes can carry out hepatocyte death. According to the Nomenclature Committee of Cell Death (NCCD), cell death can be broadly classified into regulated cell death (RCD), which can be hampered by pharmacological or genetic interventions, as opposed to accidental cell death (ACD). In 2018, NCCD published official definitions of notions linked to cell death (Table 1).

Table 1: Official notions in cell death domain. Reproduced from Ref. (29)

Box 1 Operational definitions

Accidental cell death (ACD). Virtually instantaneous and uncontrollable form of cell death corresponding to the physical disassembly of the plasma membrane caused by extreme physical, chemical, or mechanical cues.

Anoikis. Specific variant of intrinsic apoptosis initiated by the loss of integrin-dependent anchorage.

Autophagy-dependent cell death. A form of RCD that mechanistically depends on the autophagic machinery (or components thereof).

Autosis. A specific instance of autophagy-dependent cell death that critically relies on the plasma membrane Na^+/K^+-ATPase.

Cell death. Irreversible degeneration of vital cellular functions (notably ATP production and preservation of redox homeostasis) culminating in the loss of cellular integrity (permanent plasma membrane permeabilization or cellular fragmentation).

Cellular senescence. Irreversible loss of proliferative potential associated with specific morphological and biochemical features, including the senescence-associated secretory phenotype (SASP). Cellular senescence does not constitute a form of RCD.

Efferocytosis. Mechanism whereby dead cells and fragments thereof are taken up by phagocytes and disposed.

Entotic cell death. A type of RCD that originates from actomyosin-dependent cell-in-cell internalization (entosis) and is executed by lysosomes.

Extrinsic apoptosis. Specific variant of RCD initiated by perturbations of the extracellular microenvironment detected by plasma membrane receptors, propagated by CASP8 and precipitated by executioner caspases, mainly CASP3.

Ferroptosis. A form of RCD initiated by oxidative perturbations of the intracellular microenvironment that is under constitutive control by GPX4 and can be inhibited by iron chelators and lipophilic antioxidants.

Immunogenic cell death. A form of RCD that is sufficient to activate an adaptive immune response in immunocompetent hosts.

Intrinsic apoptosis. Type of RCD initiated by perturbations of the extracellular or intracellular microenvironment, demarcated by MOMP, and precipitated by executioner caspases, mainly CASP3.

Lysosome-dependent cell death. A type of RCD demarcated by primary LMP and precipitated by cathepsins, with optional involvement of MOMP and caspases.

Mitochondrial permeability transition (MPT)-driven necrosis. Specific form of RCD triggered by perturbations of the intracellular microenvironment and relying on CYPD.

Mitotic catastrophe. Oncosuppressive mechanism for the control of mitosis-incompetent cells by RCD or cellular senescence. Per se, mitotic catastrophe does not constitute a form or RCD.

Mitotic death. Specific variant of RCD (most often, intrinsic apoptosis) driven by mitotic catastrophe.

Necroptosis. A modality of RCD triggered by perturbations of extracellular or intracellular homeostasis that critically depends on MLKL, RIPK3, and (at least in some settings) on the kinase activity of RIPK1.

NETotic cell death. A ROS-dependent modality of RCD restricted to cells of hematopoietic derivation and associated with NET extrusion.

Parthanatos. A modality of RCD initiated by PARP1 hyperactivation and precipitated by the consequent bioenergetic catastrophe coupled to AIF-dependent and MIF-dependent DNA degradation.

Programmed cell death (PCD). Particular form of RCD that occurs in strictly physiological scenarios, i.e., it does not relate to perturbations of homeostasis and hence does not occur in the context of failing adaptation to stress.

Pyroptosis. A type of RCD that critically depends on the formation of plasma membrane pores by members of the gasdermin protein family, often (but not always) as a consequence of inflammatory caspase activation.

Regulated cell death (RCD). Form of cell death that results from the activation of one or more signal transduction modules, and hence can be pharmacologically or genetically modulated (at least kinetically and to some extent).

Various types of cell death like necrosis, apoptosis and necroptosis has been demonstrated to be involved in liver pathologies. Here we will focus on the apoptosis and necroptosis forms of RCD, which are under intensive investigations in liver (29).

2.1.1 Apoptosis

A distinct type of cell death, first characterized in 1972 and named apoptosis, is depicted morphologically by chromatin condensation, DNA fragmentation and membrane blebbing. Apoptosis is so important for homeostasis that conditional deficiency of one of its key factor such as caspase 8 leads to impaired heart development, while caspase 3 and 9 deficiency leads to impaired brain development. Furthermore, apoptosis is also necessary for the development of nervous and immune system during development (27). Based on the executioners and mechanism involved, apoptosis is further characterized into two distinct types (29).

2.1.1.1 EXTRINSIC APOPTOSIS

Extrinsic apoptosis is a form of RCD, dependent on caspase activity, triggered by interaction of death ligands, like TNF-α, FAS L and TRAIL, to their associated receptors present on cell surface. Extrinsic apoptosis can also occur through activation of dependence receptors in response to decreased concentration of growth factors. However, apoptosis induced by death factors is the most studied. Thus, the initial interaction between the ligand and its cognate receptors triggers the formation of death inducing signaling complex, which leads to the activation of initiator caspases-8 and -10. Cleaved caspases-8 and -10, in turn, activate the executioner caspases-3 and -7, responsible for the degradation of many cellular components, ultimately leading to apoptosis (Figure 5) (29). In type 1 cells, like lymphocytes, the amounts of caspase-8 are large enough to induce apoptosis by this extrinsic activation pathway. However, in type 2 cells, like hepatocytes, in which caspase-8 is poorly expressed, the initiation of apoptosis requires the activation of an intracellular amplification loop, corresponding to the

intrinsic pathway. In this case, the caspase-8 additionally induces the activation of the BH3 interacting-domain death agonist (BID), a pro-apoptotic member of the B cell lymphoma 2 (BCL2) family (30). BID will then activate the intrinsic pathway by interacting with the BCL2 associated X protein (BAX) to activate it (see the intrinsic apoptosis paragraph and figure 5 for more molecular events). It is important to note that activation of death receptors especially TNFR1 does not always leads to cell death.

2.1.1.2 INTRINSIC APOPTOSIS

Intracellular stressors, like DNA damage, ROS overload, damaged mitochondria or endoplasmic reticulum and cytosolic calcium overload, can induce a type of RCD, called intrinsic apoptosis. In response to stress signaling, the induction of a mitochondrial outer membrane permeabilization (MOMP) is tightly regulated by pro-apoptotic factors such as BAX and anti-apoptotic proteins like BCL extra-large (BCL-XL) members of the BCL2 family. MOMP is the most critical step of intrinsic apoptosis and is irreversible. It results in the release of apoptogenic factors such as the cytochrome c and the second mitochondria-derived activator of caspases (SMAC). The cytosolic cytochrome c can promote apoptosome formation through interaction with apoptotic protease activating factor 1 (APAF1). The formation of this complex leads to the cleavage of caspase-9, which will then target the executioner caspases-3 and -7 for their activation (Figure 5) (31).

Figure 5: Intrinsic and extrinsic pathways of apoptosis. Reproduced with permission (31)
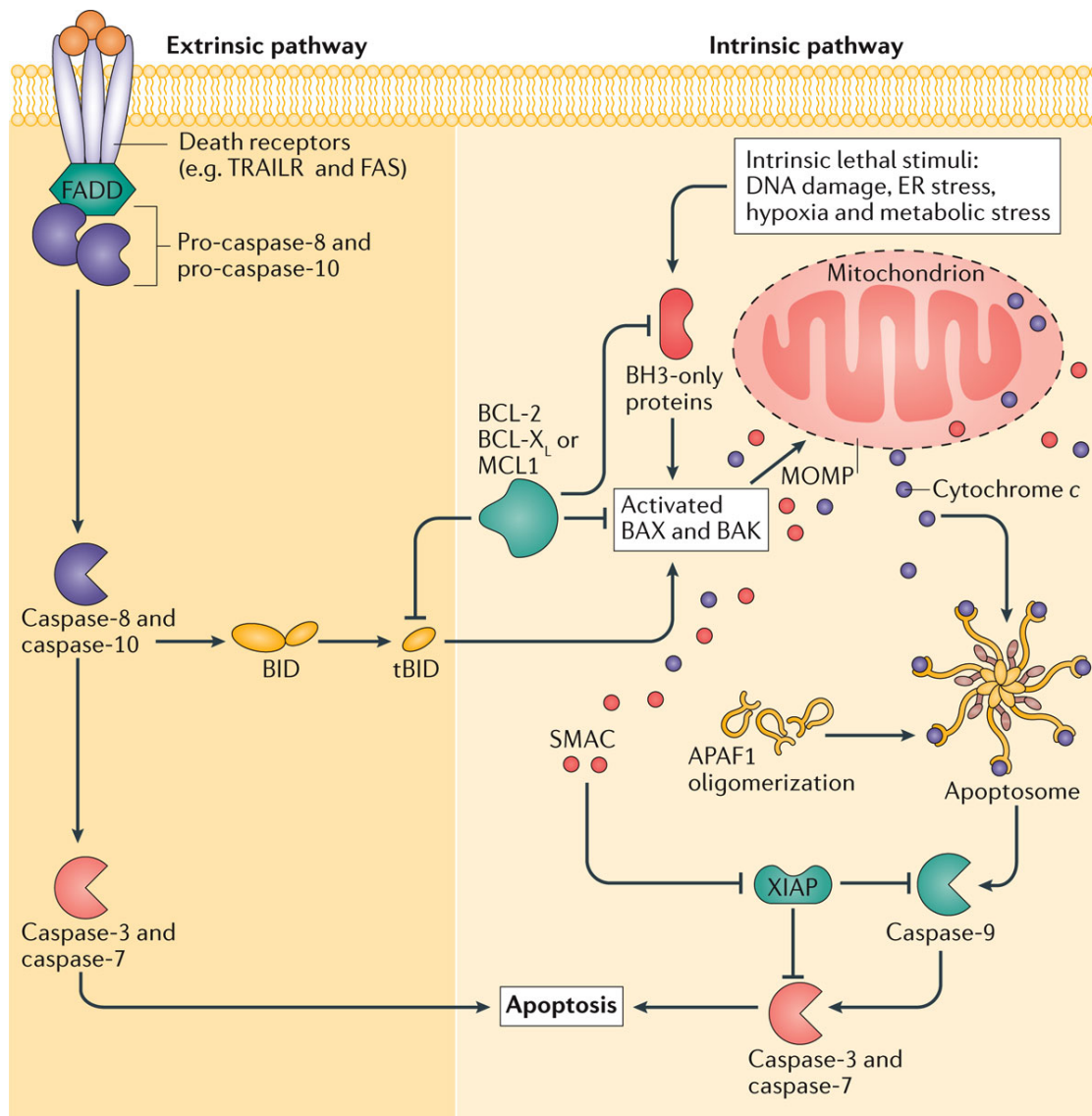
APOPTOSIS IN LIVER DISEASES

Apoptosis which can be both extrinsic or intrinsic, is involved in many liver diseases like non-alcoholic fatty liver disease (NAFLD), viral hepatitis, ischemic liver reperfusion injury, cholestatic and alcoholic hepatitis (32). Apoptosis in liver diseases can be carried out through death ligands or through different intracellular stress inducers like endoplasmic reticulum permeabilization or DNA damage further aggravating the disease (33). Cells dying by apoptosis are taken up by neighbouring cells like HSCs and KCs. Apoptotic cell bodies are indeed eliminated by phagocytosis, a phenomenon named efferocytosis (29, 33). The activation of

these phagocytic cells can lead to fibrosis or liver inflammation further progressing into more serious form, liver cirrhosis or HCC (34). Role of apoptosis can be studied through activation of executioner caspases (such as cleaved-caspase-3), pharmacologic inhibition of caspases or genetic models of mice deficient for caspases (like conditional caspase-8 knock out mice). Study of cleaved caspase-3 through immunohistochemistry (IHC) or Western blot remains an excellent tool to diagnose apoptosis (extrinsic or intrinsic). The presence of apoptosis has been demonstrated in various experimental liver pathologies such as NASH, PAMPs (LPS/D-Gal or Poly I:C/D-Gal), CCl4 induced hepatitis, HCC, alcoholic hepatitis and fulminant hepatitis (35, 36). Data from clinical trials revealed that caspase inhibition resulted in amelioration of ischemic reperfusion injury and NASH (37, 38). In mice, conditional deletion of Caspase-8 in liver parenchymal cells protected from lipopolysaccharide (LPS) and FAS induced liver injury but increased necrotic liver damage and survival in ConA induced hepatitis suggesting various role of targeting of apoptosis in various liver pathologies (39). However, inhibition of caspases can lead to activation of alternate death pathway, known as necroptosis.

2.1.2   Necroptosis

Necroptosis is another form of RCD, morphologically similar to necrosis. It is mostly carried out through interaction of receptor interacting protein kinase 1 (RIPK1), receptor interacting protein kinase 3 (RIPK3) and mixed lineage kinase domain like pseudokinase (MLKL). It can be differentiated from necrosis, which is an accidental form of cell death that cannot be controlled through pharmacological or genetic manipulations (29). Necroptosis is initiated by death receptors (DR) (TNFR1, TRAILR, FAS), Toll like receptors (TLR) (TLR3 and TLR4) or DNA-dependent activator of interferon regulatory factors (DAI) and occurs when caspases are inhibited (40, 41). In certain cells, when these conditions are met, RIPK1 and RIPK3 interact via their RIP homology interaction motif (RHIM) to form a complex that will recruit MLKL. This pseudokinase is activated and phosphorylated by RIPK3 and

subsequently translocated to the cell membrane, resulting in the rupture of the plasma membrane and the release of cellular contents, which can further potentiate inflammation. The exact mechanism by which MLKL potentiates rupture of plasma membrane remains to be elucidated but it is proposed that it modifies ions influx resulting in rupture of the cell (40, 42). The proposed mechanism of necroptosis is shown in figure 6. The role of necroptosis in liver diseases is not fully understood. It is proposed to be involved in the pathogenesis of various liver diseases, like immune mediated liver injury, alcoholic steatohepatitis, toxicant induced liver damage, NASH and Lipopolysaccharides (LPS) induced liver injury in animal models (43-48).

In vivo, evidence of necroptosis can be studied by expression and phosphorylation of constituents of the necrosome (RIPK1-RIPK3-MLKL), its formation, through pharmacological inhibitors of necroptosis (inhibitors of RIPK1, RIPK3 or MLKL) or through transgenic animal models (mice deficient for RIPK1 kinase activity, *Ripk3* or *Mlkl*) (40). Mechanism of necroptosis can be different depending on the cellular machinery and stimuli of necroptosis. In some circumstances, necroptosis would occur in a RIPK1 or RIPK3 independent manner (46, 49). Additionally, RIPK1 and RIPK3 display necroptosis independent functions (50). Therefore, necroptosis studies based on genetic or pharmacological manipulations of RIPK1 and RIPK3 should be interpreted with caution. Development of antibodies against p-MLKL paved the way for better diagnosis of necroptosis, which appears to work better in Western blot as compared to IHC. Additionally, necroptosis can be confirmed by pharmacological inhibition of MLKL to ameliorate cell viability. To explore the role of necroptosis in liver diseases, it seems to make more sense to use pharmacological or genetic manipulations targeting MLKL.

Figure 6: Execution of Necroptosis. Reproduced with permission (40)
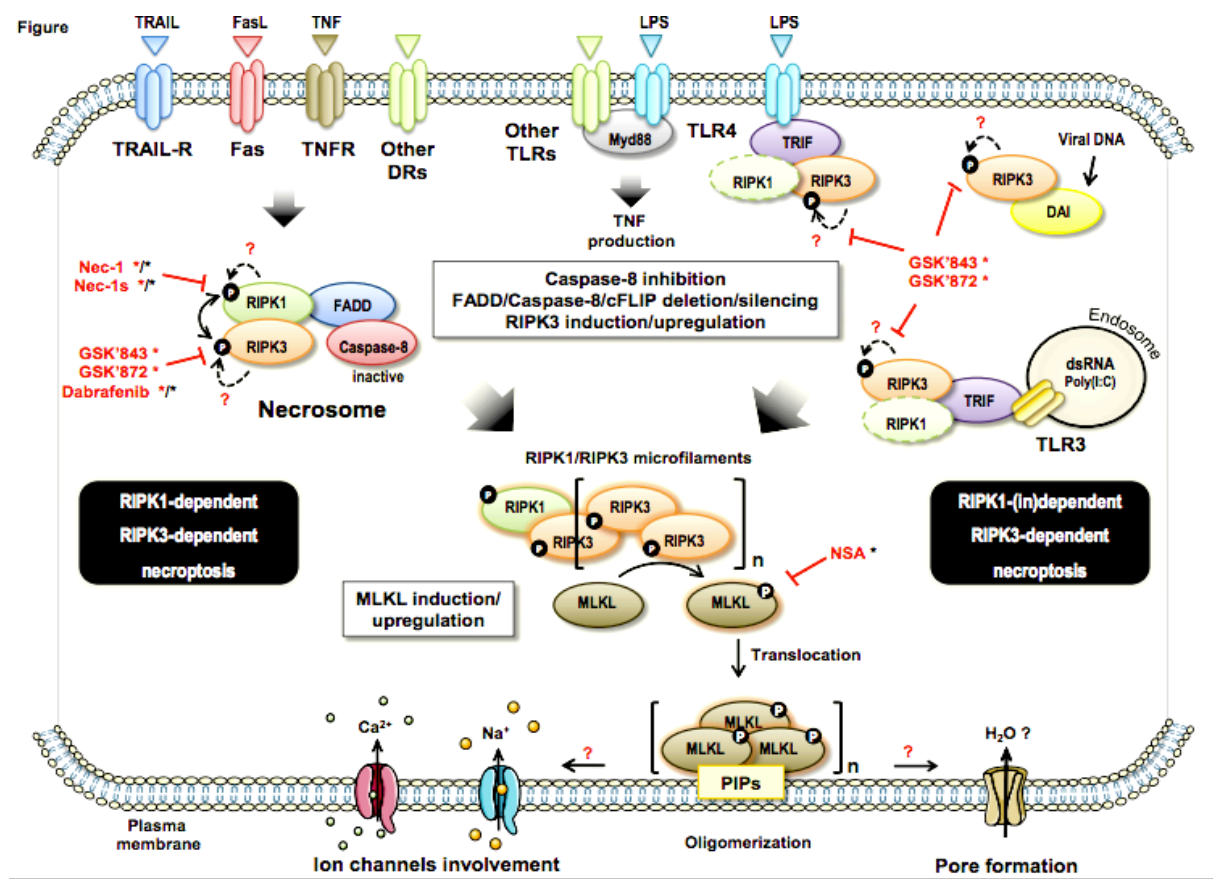
2.1.3 Tools to investigate apoptosis and necroptosis

During the last century, cell death was mainly characterized by morphological features. Historically, apoptosis was considered the only regulated form of cell death and was characterized morphologically by presence of chromatin condensation, membrane blebbing and karyorrhexis (destructive fragmentation of the nucleus). On the other hand, necrosis was considered as accidental type of cell death and was characterized by cell swelling, ruptured plasma membrane and in some cases chromatin condensation (51). Later, it was demonstrated that cell death with morphological characteristics of necrosis could be regulated pharmacologically or through genetic manipulation, called as necroptosis. Later, further types of regulated cell death with necrotic morphology were demonstrated (29, 52).

It is important to decipher the type of cell death for better understanding of pathophysiology of the disease and for the development of therapeutic agents. Necroptosis is morphologically distinct from apoptosis, but apoptosis can lead into secondary necrosis in *in-vitro* culture conditions, further complicating the identification (53). In *in-vitro* settings, cell death is measured by various tests like lactate dehydrogenase assay or through MTT, MTS or WST-1 assays. Lactate dehydrogenase is a cytosolic enzyme released when the plasma membrane is ruptured (52). MTT, MTS and WST-1 assays are based on the determination of metabolic activity (54). However, these assays demonstrate the presence or absence of cell death and fail to recognize type of cell death.

Further, use of pharmacological agents like Zvad (pan-caspase inhibitor), or inhibitors of RIPK1, RIPK3 or MLKL can ameliorate the understanding of type of cell death (55-57). Currently, Ripk3 and MLKL inhibitors are not available for mice and Nec-1s also blocks RIPK1-dependent apoptosis further hampering the identification of type of cell demise. Recently, therapeutic targeting of RIPK1, RIPK3 and MLKL coupled with Annexin-V/Propidium Iodide staining was demonstrated to differentiate between apoptosis and necroptosis (58, 59). It is important to note that Annexin-V binds with phosphatidylserine exposed on cell membrane of the dying cells and is positive for apoptosis and necroptosis. On the other hand, propidium iodide (PI) binds with nucleic acid and can only penetrate necrotic cells or late apoptotic cells. However, in late apoptotic cells, it shows small spots showing nuclear fragmentation a key characteristic of apoptotic cells. Therefore, Annexin-V and PI double negative cells are considered alive. While Annexin-V positive/PI negative cells is the characteristic of early apoptotic cells and necrotic cells are characterized by Annexin-V/PI double positive stainings. Cleaved caspase-3 and pMLKL could be evidenced by Western blot from protein cell lysates and supernatants. In *in-vitro* experiments, cell death detection assays, pharmacological inhibitors and Western blot (pMLKL and cleaved caspase-3) can be used in

combination to efficiently differentiate apoptosis and necroptosis. Some of the key differences are demonstrated in Table 2.

However, the *in-vivo* detections is more challenging. Various techniques were used to investigate cell death. Previously, TUNEL staining which characterize DNA damage was considered specific for apoptosis (60) but later on it was demonstrated that necrotic cells are also positive for Tunnel staining (61). However, apoptosis can be detected by determining cleaved caspase-3 through IHC or Western blot. Tissue positive for TUNEL and negative for cleaved caspase-3 demonstrated necrotic death including necroptosis. However, it does not confirm necroptosis. Canonic necroptosis is characterized by the necrosome formation involving RIPK1, RIPK3 and MLKL. RIPK3 determination in tissue is challenging due to some specificity issues and p-MLKL is considered as a good tool to investigate necroptosis. Recently, anti-pMLKL antibodies was shown to work in mice. However, this pMLKL determination was shown to be more effective in Western blot (61)

Table 2. Key differences in Apoptosis and Necroptosis

Technique	Apoptosis	Necroptosis
Morphology	Chromatin condensation, blebbing, cell shrinkage and DNA fragmentation	Cellular swelling and ruptured plasma membrane
Annexin-V	positive	Positive
PI	Negative in early apoptosis and positive in late apoptosis	Positive
Pan-caspase inhibitor pre-treatment	Blocks apoptosis	Can sensitize to necroptosis
Nec-1s (Ripk1 kinase activity inhibitor)	Can block only Ripk1 dependent apoptosis	Can block Ripk1 dependent necroptosis
GSK-872 (RIPK3 kinase inhibitor) work in mice and human	Does not protect	Protective
Necrosulfonamide (MLKL inhibitor) specifically work in human	Does not protect	Protective
Western Blot	Cleaved-caspase 3 positive	pMLKL positive

Non-alcoholic fatty liver disease (NAFLD) varies from simple steatosis without hepatocellular injury (NAFL) to aggressive form, the non-alcoholic steatohepatitis (NASH). NAFL is usually asymptomatic and is associated with obesity, hypertension, dyslipidemia, impaired survival and diabetes (62). NAFL is reversible and characterized by at least 5% of liver steatosis. NAFL can progress into NASH, which is characterized by hepatitis, inflammation, steatosis and cell death with or without liver fibrosis (63). NASH results in increased morbidity and mortality (64). This disease can worsen and evolve into liver cirrhosis or even into HCC. Thus, about 10-25% of patients with NASH progress in severe cirrhosis (65). Approximately one in every 10 NASH patients will develop HCC, the 3rd most common cancer. In a recent study on patients enrolled for liver transplantation in USA from 2002 to 2016, NASH was demonstrated to be the most rapidly growing cause for HCC (66). Currently, NASH is the 2nd leading cause for liver transplantation in USA after HCV chronic infection. However, NASH is predicted to takeover in next decade. It is predicted to cause huge economic losses in USA and in 4 European countries (Germany, France, Italy and UK) (67).

2.2.1 Pathogenesis of NASH

Initially, to explain the pathogenesis of NASH, Day and James proposed a two-hit hypothesis. The first hit is represented by liver steatosis, which sensitize to the 2nd hit, to promote liver inflammation, cell death and fibrosis. It is proposed that the 2nd hit includes various factors like ROS, pro-inflammatory cytokines, adipokines (68, 69). This hypothesis was too simple to demonstrate the complexity of the disease. In 2016, Buzzetti *et al* proposed a multiple-hit hypothesis to better describe the mechanism of NASH development. According to this new hypothesis, lipotoxicity, in parallel with other dietary and genetic factors, adipose tissue dysfunction, and gut microbiome regulate cell death and inflammation, which leads to the development of NASH (70). In addition to obesity, diet and environmental related factors result

in elevated serum levels of fatty acids and cholesterol, insulin resistance, proliferation and dysfunction in adipocytes as well as changes in intestinal microbiome. The insulin resistance further aggravates adipocyte dysfunction and lipolysis, resulting in the release of adipokines and pro-inflammatory cytokines (TNF-α and IL-6). These cytokines further amplify insulin resistance. Furthermore, insulin resistance can increase *de novo* lipogenesis, ultimately resulting in mitochondrial dysfunction, oxidative stress and endoplasmic stress, which further intensify inflammation. Moreover, increased intestinal permeability, genetic or epigenetic factors also contribute to the development of NASH by altering lipid metabolism or inflammation (70). Furthermore, TLR4 deficient mice resulted in amelioration of diet induced NASH suggesting the potential involvement of LPS in aggravating the liver injury (71). The proposed multiple-hit hypothesis of NASH pathogenesis is depicted in figure 7.

Figure 7: Multiple hit hypothesis of pathogenesis of NASH. Reproduced with permission (70)
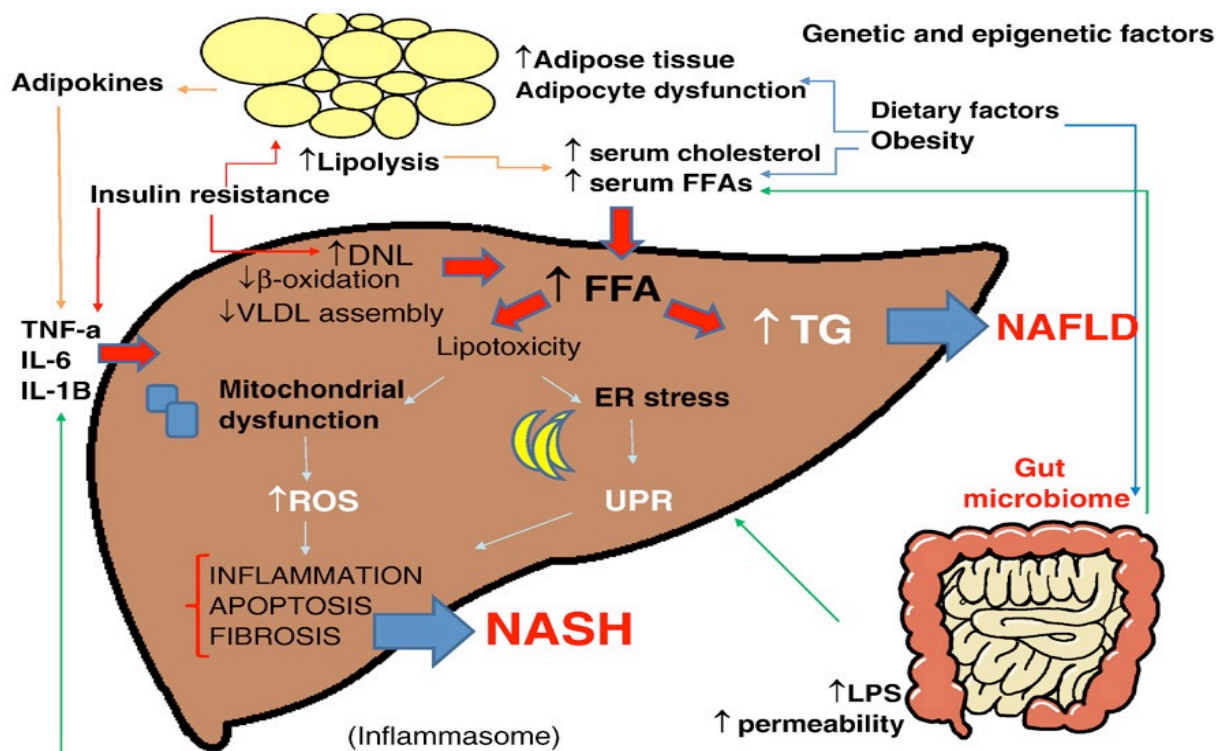
2.2.2 Risk Factors and Cause of NASH

NASH is a complex disease caused by multiple direct or indirect factors. Here we will just discuss dietary factors because of dietary models used in our research.

2.2.2.1 DIETARY FACTORS

Excessive caloric intake, from a diet that is too abundant and/or too energetic, leads to obesity and insulin resistance, well known risk factors for the progression of NASH. Diet composition, particularly in lipids and carbohydrates, is a factor influencing the progression of NASH (65).

Thus, the type of dietary lipids affects the disease (65). While omega-6 fatty acids, like linoleic acid and arachidonic acid for example, tend to stimulate inflammation, omega-3 fatty acids, like eicosapentaenoic acid, docosahexaenoic acid and α-linolenic acid, have an inhibitory effect on inflammation (65, 72, 73). There is a positive association between omega-6:omega-3 ratio and NASH progression. The decreased of this ratio, either controlled by increasing omega-3 containing foods like fish or through restriction of omega-6 containing foods, has beneficial effects in NASH resolution (65).

Consuming a lot of carbohydrates, especially fructose, increases the risk of obesity and ultimately of developing a NASH (65). This has been successfully reproduced in animal models (65). Fructose is found in large quantities in many beverages, honey, corn syrup and juice sweeteners (65). During last century, the consumption of fructose increased by 3-fold in United States (74).

Cholesterol is a key component of plasma membrane and act as a precursor for the synthesis of steroid hormones. In mammals, cholesterol requirements can be accomplished through an endogenous production. Usually, high cholesterol diets are also high in fats. Dietary cholesterol potentiates liver damage in high fat diet (HFD) induced NASH in animal models (75, 76).

2.2.3 Inflammation and inflammatory cells in NASH

Inflammation regulates progression of NAFL into NASH. Higher TNF-α and IL-6 concentrations have been reported in NASH patients as compared to healthy controls. Levels of their transcripts are more elevated in sub-cutaneous and visceral adipose tissues as compared to liver (77). These pro-inflammatory cytokines can activate immune cells, ultimately resulting in insulin resistance and metabolic dysregulation (78). The mechanism of development of insulin resistance could be due to direct or indirect interference by TNF-α in insulin signaling (79). Hepatocytes are the other cells that plays a key role in immune modulation in NASH. Indeed, hepatocyte steatosis result in cell damage leading to the release of oxidized low-density lipoproteins (OxLDL) and of DAMPs, activation factors of KCs. These resident macrophages produce various cytokines and chemokines, which recruits and activates other immune cells like neutrophils, dendritic cells and lymphocytes. KCs and neutrophils also contributes in the development of insulin resistance through release of TNF-α and elastase respectively (78). Additionally, increased intestinal permeability exposes KCs to different bacterial PAMPs further aggravating liver damage (80). Liver damage can result in the activation of HSCs, promoting liver fibrosis development (81). Trends in population of immune cells as well as their proposed functions in NASH is shown in Table 3.

Table 3. Immune cells in NASH

Immune cell	Trends in NASH	Function	Reference:
Dendritic Cells	Increases	Dendritic cells protect from fibrosis and liver damage in NASH	(12)
Kupffer cells	Increases	Depletion of kupffer cells ameliorates liver steatosis and liver injury	(82)
NK cells	increases	Depletion of NK cells resulted in increased liver damage and upregulation of genes involved in liver fibrosis	(83)
NKT cells	decreases	May have protective role in NASH progression	(84)
HSCs	increases	Results in progression of liver fibrosis	(85)
T regs	decreased	Results in amelioration of NASH	(86, 87)

2.2.4 Cell death in NASH

Cell death is the key element in NASH, which differentiates it from NAFL. This cell death is caspase-8 dependent, suggesting that apoptosis may be implied. Hepatocytes specific ablation of caspase-8 resulted in the amelioration of liver injury in NASH induced by methionine choline deficient diet (MCD) (88). Accordingly, caspase-3 depletion ameliorated liver fibrosis in MCD induced NASH (89). Furthermore, pan-caspase inhibitor treatment reduced liver injury and fibrosis, further suggesting the involvement of apoptosis in NASH induced liver injury (37, 90, 91). Higher hepatic expression of Ripk3, the key kinase involved in necroptosis, was observed in human and animal NASH. There was also an increased expression of p-MLKL, the specific executioner of necroptosis, in human and animal NASH (48, 92). Furthermore, in murine MCD-induced NASH, it was revealed that ablation of caspase-8 specifically in liver parenchymal cell (LPC) resulted in increased expression of RIPK3, suggesting the possible switch to necroptotic cell death. Additionally, combined ablation of *Ripk3* (total KO) and caspase-8 (LPC-KO) resulted in partial protection from NASH (93). In contrast to the previous findings, another study revealed the exacerbation of HFD-induced NASH in *Ripk3* knockout mice as compared to their WT controls (43). Recently, it was demonstrated that RIPK3 deficiency does not

sensitize to NASH induced liver damage, but result in increased steatosis and decreased inflammatory markers(94). Chenzu et al demonstrated that RIPK3 depletion improves NASH induced liver damage, steatosis, inflammation and oxidative stress (95). This discrepancy could be due to different models used and lack of appropriate controls since animals fed with standard chow diet were not littermates. Recently, it has been suggested that necroptosis could occur independently of RIPK3 (46). Therefore, more studies will be needed to explore the possible involvement of necroptosis in NASH.

2.2.5 Tools to investigate NASH

NASH is a complex and chronic disease requiring years to develop in human. Different interventional and non-interventional studies are used to investigate different risk or protective factors in the progression of NASH. Tools like animal models, mono culture (primary and immortalised cell lines) and co-culture are in use to better understand pathogenesis, diagnosis and treatment of NAFLD. Monocultures like primary human hepatocytes can be used to better understand the role of hepatocytes in metabolism and cell stress. However, limited availability of primary human hepatocytes, ethical hindrances, limited life on culture and absence of cell to cell interactions remains the limitation of using primary cultures(96). Maintenance of PHH can be prolonged by 3D cultures (97, 98) and immortalized cell lines (HepG2,RAW264.7) can be used to address the limited availability(99, 100). However, immortalized cell lines can have enzymatic or receptor variation. Additionally, primary hepatocytes isolated from animal origin can also be used. Cell to cell interaction can be studied by various co-cultures like co-culture of PHH with Kupffer cells or HSCs but difficult to maintain and absence of gut liver axis remains one of the limitation of co-cultures(101). Various animal models are established to mimic human NASH but so far there is not a single model to mimic exactly the human NASH. Various animal models used to study NASH induced through genetic alteration, diet induced and combination of both genetic alterations and diets induced. Every model has its own

advantageous or disadvantageous and are discussed under the heading 3.8; Animal models of NASH.

2.3 FULMINANT HEPATITIS

Fulminant hepatitis failure (FHF) is characterized by massive hepatocyte death, coagulopathy, jaundice and accompanied by hepatic encephalopathy (HE) in patients who are not suffering from chronic liver ailment (102, 103). FHF differs from acute liver failure (ALF) only by the absence of HE (104). However, sometimes ALF and FHF can also be differentiated on the basis of duration between onset of first symptoms like jaundice and HE (105-107). Various definitions used for FHF and ALF are given in Figure 8.
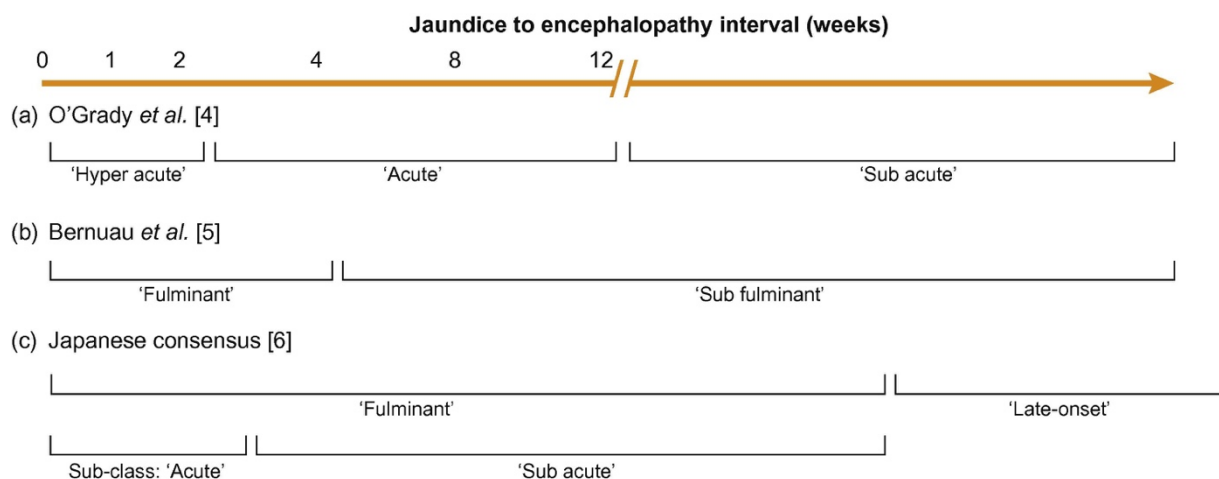
Figure 8 Schematic presentation of classification of ALF. Reproduced with permission (104).

ALF is classified according to the difference of duration (in weeks) of jaundice appearance to development of encephalopathy. According to O'Grady *et al.*, ALF is divided into hyper acute, acute and subacute phases. Bernuau *et al.* proposed fulminant and sub-fulminant hepatitis. The Japanese consensus postulated that ALF is divided into fulminant or late-onset. However, fulminant ALF can further be divided into acute and sub-acute. The duration between appearance of jaundice to encephalopathy in these classifications can be seen in figure (104)

Nonetheless, most commonly used definition of ALF includes presence of HE, with any degree of mental alteration, in patients devoid of pre-existing liver cirrhosis, and duration of liver failure is less than 26 weeks (108). Additionally, it may include patients with vertically transmitted HBV infection, auto-immune hepatitis or Wilson disease in spite of the potential induction of liver cirrhosis, provided that duration remains less than 26 weeks (109).

Fulminant hepatitis is caused by different viruses, autoimmune diseases, drug induced liver injury, idiosyncratic, metabolic and vascular diseases (110). The various etiologies of fulminant hepatitis are shown in Table 4. Drug induced liver injury and viral hepatitis are the leading causes of FHF in developed and developing nations, respectively (104, 111).

Table 4: Etiology of Fulminant hepatitis. Reproduced with permission (112)

Viral	**Hepatitis A,B**,C,**D**,E, CMV HSV, EBV, VZV, HHV 6, Parvo-virus B19, Parainfluenza, Yellow Fever, and others
Idiosyncratic	Halogenated hydrocarbons, **Coumarins**, Methyldopa, Phenytoin, **Carbamazepin, Valproic acid**, Rifampicin, Penicillin, Sulfonamides, **Chinolones**, etc.
Toxic Dose-dependent	**Acetaminophen (Paracetamol)**, Isoniazid, Tetracycline, Methotrexat, Carbon tetrachloride, Amphetamins, **Amanita phalloides-Toxin**
Toxic synergistic	Ethanol + Acetaminophen, Barbiturate + Acetaminophen, Isoniazid + Rifampicin
Metabolic	**M. Wilson**, alpha-1-AT-deficiency, Galactosemia, Tyrosinemia, Reye-Syndrome, NASH
Associated with pregnancy	Acute fatty liver of pregnancy, HELLP-Syndrome
Vascular	**Budd-Chiari-Syndrome**, veno-occlusive disease, shock, heart failure
Miscellaneous	**Autoimmune-hepatitis**, malignant infiltration, hyperthermia, sepsis

2.3.1   Viral Fulminant Hepatitis

Viral hepatitis is the leading cause of FHF in Western world. Even if non-hepatitis viruses, such as the herpes simplex virus (HSV) or the cytomegalovirus (CMV), are occasional etiological factors in immunocompromised patients, most fulminant hepatitis with an identifiable viral cause originates from infection with hepatotropic virus, mainly the hepatitis B virus (HBV) and

more rarely the hepatitis A, C or E viruses (HAV, HCV, HEV) (108, 113). Orthotopic liver transplantation remains the leading life-saving option for patients with fulminant hepatitis (114, 115).

3   MURINE MODELS AS A TOOL TO STUDY ACUTE HEPATITIS

Understanding the pathophysiology of a disease is pivotal for diagnosis and to devise treatment strategies. Murine models are widely used to mimic the pathophysiology of human liver diseases. They are preferred because of certain advantages such as pathophysiological mimicry of human diseases, limited costs, ease of handling, short life cycle, short gestation, easy availability, minimal risk to manipulators, availability of genetically-modified strains etc. Furthermore, drug approval for human usage necessitates prior animal testing. The mouse shares 99% of its genome with human. Different mice models have been developed to study acute or chronic hepatitis mimicking various types of human hepatitis.

3.1   CARBON TETRACHLORIDE (CCL$_4$) INDUCED ACUTE HEPATITIS

Carbon tetrachloride (CCl$_4$) is an organic compound, which was widely used as precursor to refrigerants, cleaning agents, degreasers and as a constituent in fire extinguishers until its hepatotoxic and carcinogenic potential was elucidated. Once metabolized in liver, cellular cytochromes convert it into trichloromethyl radical (CCl$_3$), which binds more to cellular proteins, lipids and nucleic acids and interferes with lipid metabolism, leading to steatosis. In the presence of oxygen, CCl$_3$ can further be oxidized into trichloromethyl peroxy free radical (CCL$_3$OO•), which causes cellular oxidative stress ultimately increasing cell permeability and release of cellular calcium leading to cell death (116).

CCl$_4$ also activates a plethora of inflammatory cytokines such as TNF-α, nitric oxide and transforming growth factor (TGF) α and β (116). TNF-α is a pro-inflammatory cytokine which binds to its cognate receptors TNFR1 and TNFR2, although its major functions are carried out

through TNFR1 binding. In physiological conditions, once bound to TNFR1, the NFκb pathway is activated, which results in the induction of anti-apoptotic genes, ultimately promoting cell survival. When protein synthesis is inhibited, as in case of CCl_4, TNF-α can promote cell death. TNFR1 ablation protects from CCl_4 induced hepatotoxicity, suggesting the involvement of TNF-α induced cell death (117). Neutralization of TNF-α can be protective or damaging in this model, depending on doses of TNF-α neutralizing agent (118). These finding may be due to the potential involvement of TNFR2 in the protection of CCl_4-induced hepatotoxicity (116).

CCl_4 can be administered intraperitoneally, subcutaneously or orally to induce liver damage. Single shot of CCl_4 can induce acute hepatitis after 12-24 hours depending on the dose and route of administration (118-120). Furthermore, the level and course of hepatotoxicity depends on the age, sex, dose and route of administration of CCl_4 and presence of other potentiating agents (118-121). This is an excellent model to study hepatotoxicant-induced liver damage.

3.2   ACETAMINOPHEN INDUCED ACUTE HEPATITIS

Acetaminophen (paracetamol or N-acetyl-p-aminophenol; APAP) is a widely used non-steroidal anti-inflammatory drug which is listed under the category of "over the counter medicines". Indeed, APAP is most widely used as antipyretic and analgesic in USA. Generally, FDA-recommended dose up to 4 grams daily is not injurious. Higher doses can lead to FHF. (122).

APAP-induced hepatoxicity is the leading cause of acute liver failure in developed countries. Its severity depends on various factors like age, sex, nutritional status, genetic background and functioning of liver (122). Acetaminophen pathophysiology in mice is an excellent tool to ameliorate the understanding of human APAP induced hepatotoxicity.

At therapeutic doses, APAP is metabolized into nontoxic glucuronide and sulfate conjugates while at higher doses, it is metabolized by cytochrome P450 to an active toxic metabolite N-

acetyl-p-benzoquinone (NAPQI). This NAPQI is detoxified by glutathione (GSH). At toxic

doses, GSH activity is saturated. Remaining active metabolite binds to the cysteine sulphydral

group of proteins leading to oxidative stress and finally to the rupture of mitochondrial

membranes. Furthermore, the c-Jun N-terminal kinase (JNK) is activated through various

kinases like RIPK1, mixed lineage protein kinase 3 (MLK3), protein kinase C (PKC), glycogen

synthase kinase 3 beta (GSK3 β), apoptosis signal-regulating kinase 1 (ASK1) and mitogen-

activated-protein-kinase kinase 4 (MKK4). The activated JNK stimulates SH3 homology

associated binding protein (Sab), ultimately resulting in amplification of ROS production

causing mitochondrial permeability transition (123, 124). The released contents like

endonuclease G and apoptosis inducing factor results in DNA fragmentation. The

mitochondrial membrane dysfunction and DNA fragmentation are the main causes of necrotic

cell death (125, 126).

The role of TNF-α, TNFR1 and KCs in APAP-induced hepatotoxicity remains controversial

(127-132). The conflicting data could be due to differences in dose, slaughtering time after

injection, strain variation, different method of KC depletion or it could be due to microbiota

disparities in gut liver axis (125). Lack of cleaved caspase-3 in liver lysates suggests the absence

of apoptosis (133).

Pharmacological inhibition of RIPK1 kinase activity or transient knockdown of RIPK1 result

in amelioration of APAP-induced hepatotoxicity while *Ripk3* and *Mlkl* deficiency does not

improve the liver damage (134). These findings suggest that APAP-induced liver damage is

independent of necroptosis. However, APAP-treated *Ripk1*[LPC-KO] mice (mice deficient for

Ripk1 specifically in liver parenchymal cells) displayed similar level of transaminases as

compared to their wild type treated littermates suggesting that RIPK1 is dispensable in APAP-

induced hepatotoxicity (135). The discrepancy in the role of RIPK1 in APAP toxicity could be

due to the different methods of depletion of RIPK1. In *Ripk1*[LPC-KO] mice, adult animals never

expressed RIPK1 in LPCs, while *Ripk1* was depleted transiently in all cells in anti-sense oligonucleotide (ASO) treated mice. Therefore, potential involvement of compensatory expression and contribution of RIPK1 present in non-parenchymal cells (NPC) cannot be ruled out. Further investigations will be needed to better understand the role of RIPK1 in APAP induced hepatotoxicity.

3.3 ALCOHOLIC LIVER DISEASE

Alcoholic liver disease (ALD) is one of the leading cause of liver related mortality in occidental countries. Ethanol is metabolized in liver into acetaldehyde, which further results in production of ROS. Furthermore, activation of NADPH oxidase in KCs also results in ROS generation. These ROS and subsequent inflammation lead to steatosis, oxidative stress and ultimately to cell demise (136).

There are different protocols proposed to induce alcoholic disease in mice (137). The more relevant model mimicking human pathology is the National institute on alcohol abuse and alcoholism (NIAAA) model of alcoholic hepatitis (138). In this method, ALD is induced in mice through alcoholic diet feeding for 10 days and a short binge of higher dose of alcohol is given afterwards. However, the level of liver damage is limited as compared to human ALD (136, 139). Extent of liver injury induced in ALD depends on various factors like genetic background of mice, sex, environment of the animal facility and dose of ethanol used (137). In rat, increased intestinal permeability during ALD induces increased bacterial LPS leakage, further aggravating the disease (140). In agreement with this study, depletion of TLR4 in rats resulted in amelioration of ALD further suggesting the potential role of LPS (Uesugi 2003). Similarly, LPS injection exacerbates the ALD in mouse by induction of inflammatory cytokines like TNF-α through KCs (141). TNFR1 knockout mice or neutralization of TNF-α improve the ALD (142), underlying the role of TNF-α in ALD morbidity.

ALD can induce hepatocyte death via apoptosis, necrosis or necroptosis (143). Level of liver RIPK3 is elevated in alcoholic patients and *Ripk3* depletion in mice ameliorated the ALD (44, 144). Inhibition of RIPK1 kinase activity by single injection of Nec-1s before binge does not improve liver injury but improves the inflammation in NIAAA model of alcoholic liver injury (144).

3.4   LPS/D-GALACTOSAMINE INDUCED TOXICITY

LPS, also called bacterial endotoxins, are a constituent of bacterial outer membrane. LPS can induced endotoxic shock in human but the mice are generally resistant to LPS. Very high doses are required to induce septic shock in mice (145). LPS, a PAMP, is recognized by pathogen recognition receptors such as TLR4 present on KCs. Its activation results in the production of a plethora of inflammatory cytokines in 1-2 hours post LPS-administration. LPS alone does not induce liver damage in wild type mice. Pre-administration of D-galactosamine (D-gal), which blocks the NF-κb pathway, sensitizes mice to LPS induced liver damage (145). LPS/D-Gal induced liver damage is mainly through TNF-α and its cognate receptor TNFR1 (146). Caspase inhibition results in the amelioration in LPS/D-Gal induced liver injury, suggesting the involvement of apoptosis (147). In parallel, RIPK1, RIPK3 and p-MLKL protein expressions are increased after LPS/D-Gal treatment. Thus, cell death by necroptosis could also be expected. Furthermore, chemical inhibition of RIPK1 kinase activity by Nec-1 pre-treatment ameliorates the LPS/D-Gal induced liver damage (148). However, use of Nec-1 has specificity issues that necessitates confirmation with the more specific Nec-1s RIPK1 kinase inhibitor.

3.5   POLY I:C/D-GAL INDUCED ACUTE HEPATITIS

Polyinosinic:polycytidylic acid (Poly I:C), is a synthetic double stranded RNA, which is used as viral double stranded RNA mimetic and is mainly recognized by the pathogen recognition TLR3 present at cell surface and in intracellular endosomes (149). Poly I:C can also bind to

cytoplasmic receptors like RIG-1, MDA5 and PKR (150). Once bind to the endosomal receptor TLR3, it stimulates antiviral response through production of type 1 interferons (IFN-α and IFN-β), and also activates the NF-κb pathway which results in induction of inflammatory cytokines such as TNF-α, IL-6 and IL-12 (151). Pretreatment by D-Gal sensitizes to Poly I:C induced hepatitis. Neutralization of TNF-α protects from Poly I:C induced hepatitis underlying the main role played by this cytokine in this model (152). Furthermore, downstream of TLR3, TRIF can lead to interferon production or can interact with RIPK1, resulting in cell survival through NF-κb pathway activation, or in cell death through apoptosis or necroptosis (153, 154). This model is a good tool to investigate immune mechanisms occurring during viral hepatitis.

3.6   MHV3 INDUCED HEPATITIS

Murine hepatitis virus (MHV) is a positive sense single stranded RNA virus belonging to the *coronaviridae* family, the largest known RNA viruses. It can cause enteritis, hepatitis and demyelinating encephalitis in susceptible rodents. The type of strain used determines the type of pathology, like MHV type 3 (MHV3) causes severe hepatitis. Liver damage caused by MHV3 depends on several factors including route of inoculation, viral dose and also age, strain and immune status of the infected rodents. It causes death of mice within 3-5 days after inoculation (155).

In the liver, the cell tropism of MHV3 results in the infection of resident macrophages (KCs), liver sinusoidal endothelial cells, hepatic stellate cells and hepatocytes (156). Host-pathogen interactions cause release of high levels of inflammatory mediators while repressing the production of immunosuppressive factors (157-160). Concomitantly, activated macrophages and sinusoidal endothelial cells secrete the prothombinase fibrinogen-like protein 2 (FGL2), initiating fibrin matrix formation. The induced coagulopathy disrupts blood supply, ultimately

leading to death of hepatocytes (161). This model mimics the pathophysiology of human fulminant viral hepatitis and is a good tool to investigate the disease.

3.7 CONCANAVALIN INDUCED HEPATITIS

Concanavalin A (ConA) is a plant lectin extracted from jack beans. Intravenous administration in rodents triggers an hepatitis and is widely used to mimic human autoimmune hepatitis (162). Administration of ConA leads to the presentation of ConA by KCs and LSECs through mannose receptors, inducing the secretion of different cytokines like TNF-α and IFN gamma. The binding of TCR to ConA presented through KCs and LSECs leads to the activation of T cells and NKT cells, which further leads to a burst of inflammatory cytokines like TNF-α, IL-2, IL-4, IL-6, IL-12 and IFN gamma as well as anti-inflammatory cytokines like IL-10 (126, 163, 164). Mice deficient for NKT cells are resistant to ConA induced liver injury (165). The peak plasma concentration of these cytokines varies, but it is within 6 hours post administration of ConA and starts to decrease after (164). The susceptibility of mice to ConA-induced hepatotoxicity depends on dose of ConA used, genetic background and sex of the mice (163). There is massive cell death carried out through death receptor activation like TNFR1, FAS and TRAIL receptor 1 & 2 by their respective death ligands (165-171). Therefore, neutralization of these death ligands results in the amelioration of liver injury (166-168, 172-174). Various studies reveal the potential involvement of necroptosis in this model as blocking the Ripk1 kinase activity through genetic modification or chemical inhibition reduced the liver damage (167). Interestingly, a study reveals that this necroptosis is possibly carried out in Ripk3-independent manner (46).

4   ANIMAL MODELS OF NASH

The ideal model to replicate human pathophysiology of NASH, would combine obesity, insulin resistance, inflammation, cell death, hepatomegaly, steatosis. Different dietary and genetic animal models are in use to try to replicate human NASH. Currently, there is no ideal model to mimic human NASH. Each model has their own advantages and disadvantages.

4.1   ANIMAL MODELS WITH GENETIC INTERVENTIONS

Different epidemiological studies in human reveal an association between leptin gene mutation and NASH. Animal models with leptin deficiency *ob/ob* or deficiency of leptin receptor *db/db*, develop altered feeding behavior, decreased activity, obesity, steatosis and insulin resistance. These mice will develop NASH only under specific diets such as high fat diet (HFD), MCD or after administration of endotoxins (101, 175, 176). Mice with mutations in agouti gene and melanocortin 4 receptor also spontaneously develop steatosis, obesity and insulin resistance, but an additional specific dietary intervention is required for NASH development (177-180).

4.2   DIETARY MODELS

Various human epidemiological studies have shown that factors for NASH development could be diets high in fat, cholesterol or in sugars, especially lipogenic sugars like fructose. Dietary models are the most common models used in NASH research. In dietary mouse models, NASH is induced through diets rich in fats, cholesterol or sugars. Furthermore, it can also be induced by feeding animal with diets deficient for methionine and choline, nutritional element involved in lipid metabolism. Deficiency of methionine or choline in the diet increase hepatic intake of lipids and decreased secretion of VLDL resulting in steatosis and development of NASH (181).

As NASH is shown to be associated with diabetes, an animal model, named STAM™, was also developed by feeding diabetic mice with an HFD. Diabetes is induced by subcutaneous streptozotocin injection two days after birth. All males develop HCC at 16 weeks post HFD

feeding (182). The development of diet-induced NASH can be accelerated by addition of certain toxicants such as CCl$_4$ (183, 184). A short summary of the pathophysiological findings in different NASH models is given in table 5.

Table 5. Summary of pathophysiological findings in various animal models of experimental NASH

Model	Obesity	Insulin Resistance	Hepatomegaly	NAFL	NASH	Fibrosis	Reference:
MCDD	-	-	+	+	+	+	(185)
CDAA	-	-	+	+	+	+	(186)
HFD	+	+	+	+	+	+	(187)
Atherogenic	-	+	+	+	+	+	(188)
Western Diet	+	+	+	+	+	+	(189)
ob/ob	+	+	+	+	-	-	(190)
db/db	+	+	+	+	-	-	(191)
ob/ob + MCD	+	+	+	+	+	-	(192)
db/db+ MCD	+	+	+	+	+	+	(192)
STZ + HFD	-	+	+	+	+	+	(182)
CCl4+CDAA	+	+	+	+	+	+	(193)
CCl4+Western Diet	-		+	+	+	+	(184)

5 RECEPTOR INTERACTING PROTEIN KINASE 1 (RIPK1)

The RIP kinase family consists of seven members (RIPK1-7), which are characterized by the presence of homologous kinase domain (KD) (194). The different kinases are characterized by different functional domains. The first identified member of the family, RIPK1 was discovered in 1995 (195). Since its discovery, lot of research works were done to explore the structure and function of RIPK1 both *in vitro* and *in vivo*. In addition to kinase family signature (N-terminal KD), RIPK1 also contains a C-terminal death domain (DD) and a bridging intermediate domain

(ID) that also harbors a RIP homotypic interaction motif (RHIM) (49, 196). The DD of Ripk1 interacts with death ligand receptors like TNFR1, TRAIL-R1&2, FAS and other DD containing adaptor proteins like Fas associated protein with DD (FADD) and TNF receptor associated DD (TRADD). Auto-dimerization of DD of RIPK1 may activate its kinase activity to regulate apoptosis or necroptosis (197). Furthermore, through ID, it interacts with RIPK3, Mitogen activated protein kinase kinase 1 (MEKK1), Mitogen activated protein kinase kinase 3 (MEKK3) and TIR-domain-containing adapter-inducing interferon Beta (TRIF) (194, 196, 198). The proposed schematic diagram of RIPK1 is shown in figure 10. To date, it is the kinase portion of RIPK1, which is currently under research as a potential pharmacological target in treating experimental liver pathologies.

Figure 9: Schematic diagram of RIP kinase family. Reproduced with permission (194)
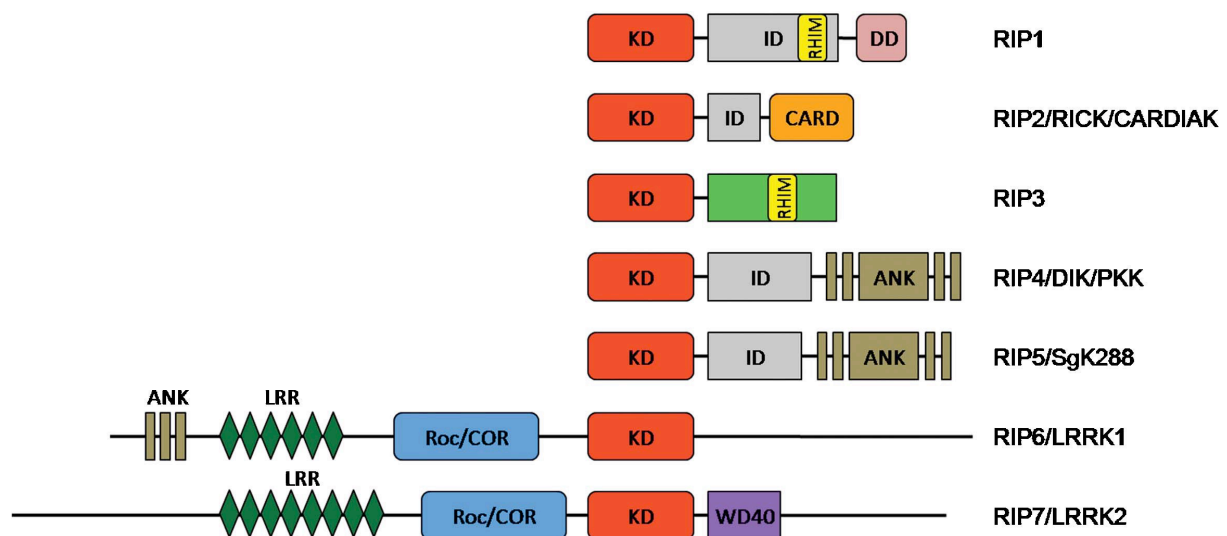
Ripk1 decides the fate of cell, whether to survive or to die by caspase dependent apoptosis or RIPK3/MLKL dependent necroptosis. It plays key role in response to various death stimuli like death ligands (TNFα, FAS L and TRAIL), PAMPs (bacterial LPS and viral dsRNA), interferons, DAI, T cell receptor (TCR), DNA damage, stresses including but not limited to calcium overload, endoplasmic reticulum stress (153, 198-205).

Figure 10: Schematic diagram of Ripk1. Reproduced with permission (49)
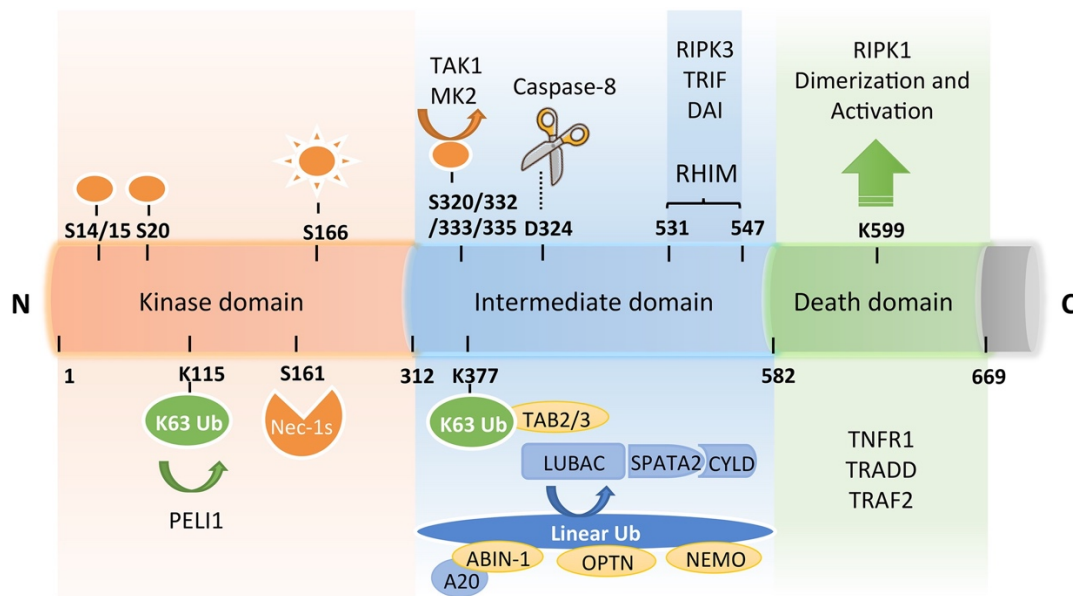
5.1 CELL SURVIVAL SIGNALING

Nuclear transcription factors are key regulators in cell survival, cell stress, differentiation and cancer development. In mammals, the NF-κB family of transcription factors contains five members, i.e. Rel A (p65), Rel B, c-Rel and precursor proteins NF-κB1 (p105) and NF-κB2 (p100) which are ultimately converted into p50 and p52, respectively. This ultimate conversion is carried out through two different pathways of NF-κb activation, i.e. canonical and non-canonical pathway (206, 207).

The canonical pathway is activated by TNF-α, FAS L, TRAIL, PAMPs, while the non-canonical pathway is activated downstream of CD40 ligand, BAFF and Lymphotoxin Beta ultimately resulting in the nuclear translocation of p52 as compared to p50 and p65 in the canonical pathway (shown in figure 11) (206).

Figure 11: Canonical and non-canonical pathways of NF-κb activation. Reproduced with permission (206)
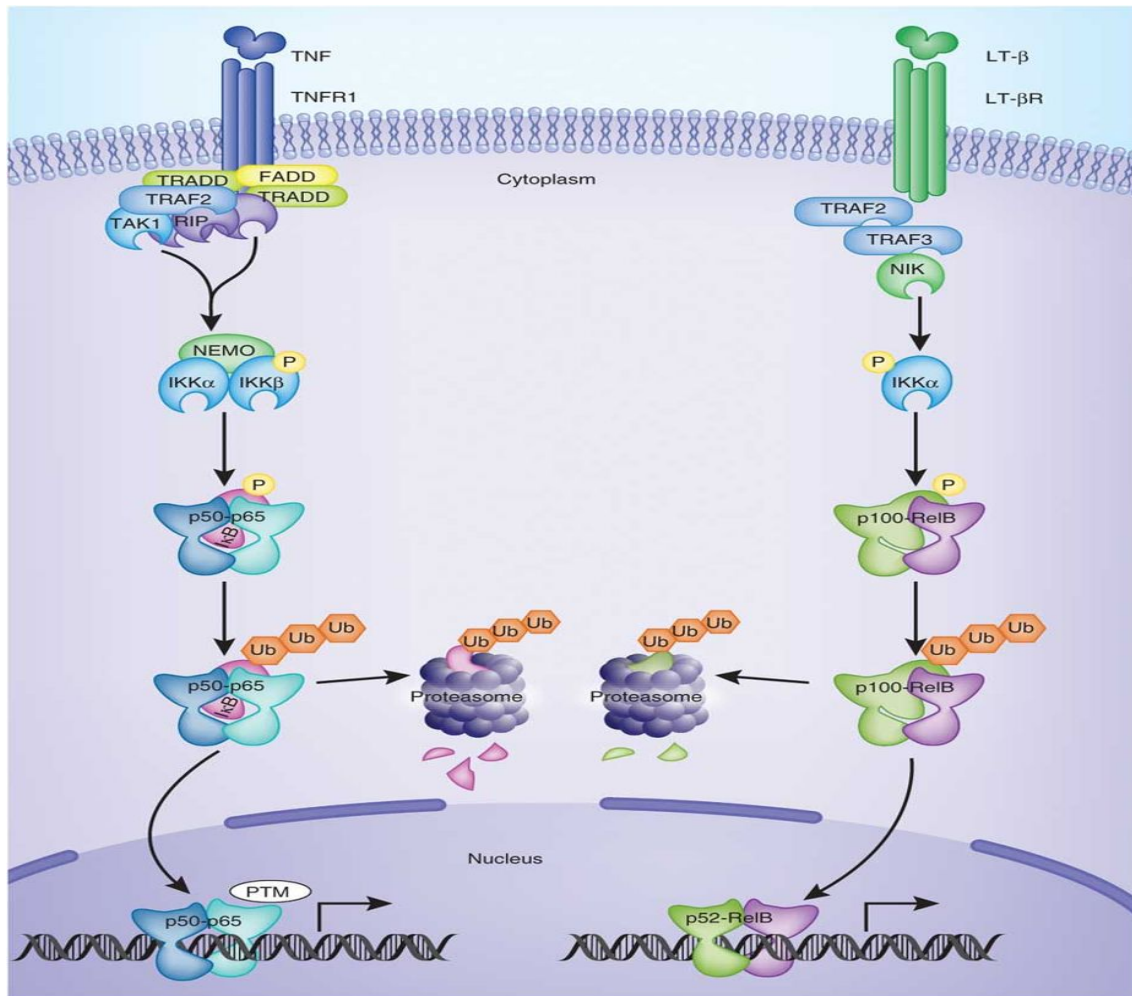
It is the canonical pathway downstream of TNFR1 signaling, which is under extensive scientific discussions. When TNF-α, a pleotropic cytokine, binds to TNFR1, which results in its conformational modification, the receptor recruits TRADD and RIPK1. Later, TRADD serves as a platform to recruit TNF receptor-associated factor 2 (TRAF2) and/or TNF receptor-associated factor 5 (TRAF5), which facilitates binding of cellular inhibitor of apoptosis 1 (cIAP1) and cellular inhibitor of apoptosis 2 (cIAP2) to the complex, ultimately resulting in ubiquitination of RIPK1. The ubiquitinated RIPK1 is thought to recruit transforming growth factor beta-activated kinase 1 (TAK1), TAK1 binding protein 2 (TAB2), TAK1 binding protein 3 (TAB3) and IκB kinase (IKK) complex (IKK-α, IKK-β and NF-κB essential modulator

(NEMO) also known as IKK gamma). This is called complex I as shown in Figure 12. This induces the phosphorylation and proteasomal degradation of IκB, which results in the release of the NF-κB dimer p50-p65. Then, the NF-κB dimer translocate into nucleus to upregulate inflammatory and anti-apoptotic genes (49, 206, 207). NF-κB induced anti-apoptotic proteins like cFLIP promote survival by inhibition of apoptosis and necroptosis (Figure 12) (208).

Figure 12: TNFR1 signaling in apoptosis and necroptosis. Reproduced with permission (49)
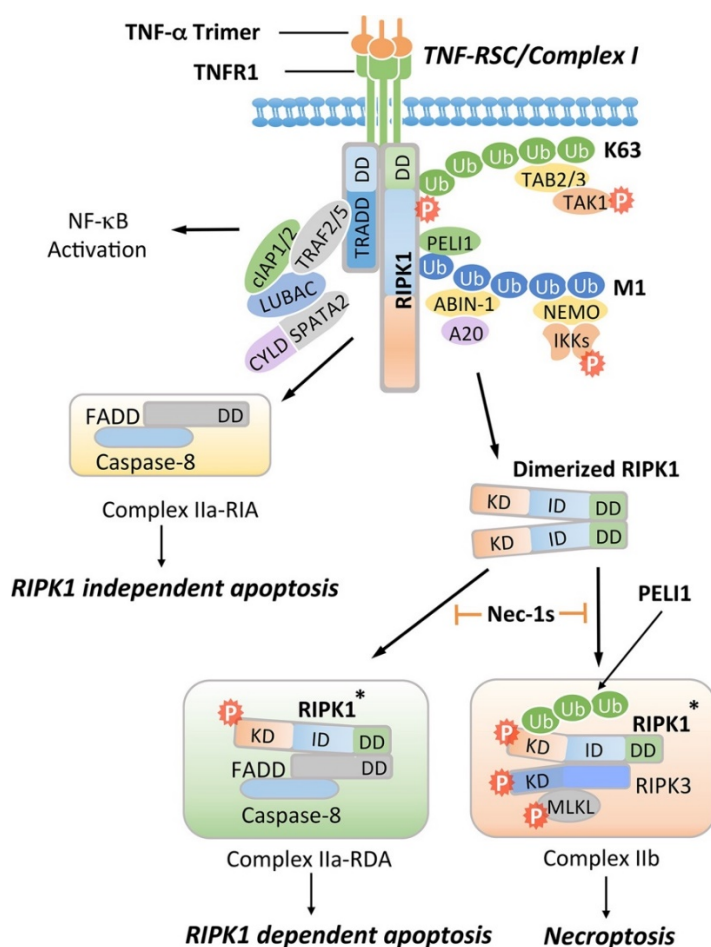
5.2 CELL DEATH SIGNALLING

TNFR1 signalling leads to the formation of another cytosolic complex called complex II, which is mainly composed of Ripk1, FADD, Casp-8 and cFLIP (209, 210). However, this complex can additionally recruit Ripk3 depending on the type of tissue. Despite the formation of this complex II, TNFR1 signalling results in cell survival due to the presence of multiple

checkpoints (209, 210). These checkpoints can be early (transcription-independent) or late (transcription-dependent). The early checkpoints, or transcription-independent checkpoints, are regulated by the components of complex I and late checkpoints, or transcription-dependent checkpoints, are regulated by NF-κb (207, 209, 210). Site-specific ubiquitination and phosphorylation of Ripk1, regulated by various components of complex I like cIAP, LUBAC, MK2, TAK1, Nemo play key role in cell fate (211-216). Roles of this post-translational modifications of Ripk1 in regulating cell survival and cell death is discussed elsewhere (207, 210). The pharmacological or genetic intervention mediating inhibition of NF-κb-pathway or disruption of the complex I result in the activation of death signalling. Death can be induced through Ripk1-independent apoptosis (Complex IIa RIA), Ripk1-dependent apoptosis (Complex IIa RDA) or necroptosis (Complex IIb) as shown in figure 12 (49). NF-κb signalling inhibition, by D-Galactosamine (hepatocyte specific transcriptional inhibitor) or by cycloheximide (translational inhibitor), interferes with the anti-apoptotic signalling and results in the formation of complex IIa RIA (Ripk1-Independent Apoptosis). This complex is formed by the interaction of caspase 8 with FADD ultimately resulting in Ripk1-independent apoptosis (49, 217). Furthermore, pharmacological or genetic intervention resulting in inhibition of IAPs or deficiency of TAK1, IKK/NEMO or linear ubiquitin chain assembly complex (LUBAC) results in the activation of complex IIa RDA (Ripk1-Dependent Apoptosis) (49, 218, 219). This complex is formed by the interaction of Ripk1 with FADD and caspase-8, which mediates Ripk1-dependent apoptosis (49). In complex IIa RDA, it has been demonstrated that caspase-8 cleaves Ripk1 resulting in the inhibition of necroptosis. Therefore, pharmacological inhibition or genetic deficiency of caspase-8 results in the formation of complex IIb that leads to the interaction of Ripk1 with Ripk3. This complex then activates MLKL by its phosphorylation, ultimately resulting in necroptosis (219). Recently it has been demonstrated that MLKL is phosphorylated in nucleus and then translocated to cytoplasm (220). In cytoplasm, oligomers

of p-MLKL bind to phosphatidylinositol phosphates in plasma membrane and ultimately resulting in membrane permeabilization (221-223). Phosphorylation of Ripk1 regulated by TAK1 plays key roles in cell fate. Indeed, transient and sustained phosphorylation of RIPK1 by TAK1 leads to apoptosis and necroptosis respectively (219). Both, Ripk1-dependent apoptosis and necroptosis can be inhibited by pre-treatment with Nec-1 (55). Mice deficient for RIPK1 kinase activity develop normally, suggesting that RDA and RIPK1 dependent necroptosis are not essential for liver homeostasis. (49).

5.3 RIPK1 IN TLR SIGNALLING

There are specific molecular receptors called pattern recognition receptors (PRRs) which are responsible for recognition of microbial invasion. These PRRs can be present on cell surface, in endosomal membranes, and in cytoplasm or in the serum. They recognize PAMPs and DAMPs, respectively issued from microorganisms and damaged tissue. These PRRs includes but not limited to NOD-like receptors, RIG-I-like receptors and Toll-like receptors (TLRs). Below, we briefly discuss about TLRs and the role of RIPK1 downstream of TLRs (224, 225).

5.3.1 Toll like receptors (TLRs)

TLRs are type of PRRs, characterized by extracellular leucine-rich repeats, transmembrane domain and cytoplasmic domain. TLRs recognize various associated PAMPs and play key role in activation of the innate immune response. In mammals, there are 10 known types of TLRs (TLR1-10), while in mice, 12 TLRs (TLR1-9 and TLR11-13) has been characterized so far (226, 227). Each type of TLR binds with their respective PAMPs and can be found on cell surface or in the endosome (228). Type of TLRs and their respective PAMPs and their location is given in Table 6. Once bound to their respective PAMPs or DAMPs, TLRs function in myeloid differentiation primary response 88 (MyD88)-dependent and MyD88-independent pathways (229). All TLRs, except TLR3, function through MyD88 dependent pathway, while

TLR3 function through binding with TRIF (226). TLR4 is the only TLR, which can function through both pathways (226). Below, TLR signalling with specific reference to TLR4 and TLR3 will be briefly presented.

Table 6. Type of TLRs and their cognate receptors

Type	Location	Cognate molecules	Reference:
TLR1	Cell surface	Bacterial lipoproteins such as outer surface protein A(OspA) of Borrelia	(230, 231)
TLR2	Cell surface	It forms heterodimers with TLR1 and TLR6 and recognizes various PAMPs originated from bacteria, virus, protozoa and fungus	(232)
TLR3	Endosome/cell surface	Viral dsRNA	(233, 234)
TLR4	Cell surface	Bacterial LPS	(230)
TLR5	Cell surface	Flagellin	(224)
TLR6	Cell surface	Diacyle lipopeptides from Mycobacterium	(224)
TLR7	Endosome	Bacterial or Viral RNA	(224)
TLR8	Endosome	RNA Viruses	(224)
TLR9	Endosome	Bacterial and Viral DNA, hemozoin	(235)
TLR10		Ligands from Listeria and influenza virus	(236, 237)
TLR11	Endosome	Flagellin	(238)
TLR12	Endosome	Profilin originated from toxoplasma	(239)
TLR13	Endosome	Bacterial 23S rRNA	(240)

In MyD88-dependent pathway, once TLR4 bind with LPS through LPS binding protein, CD14 and MD2, it goes through conformational modification resulting in activation of MyD88 protein indirectly through TIR domain containing adapter protein (TIRAP) (241). Activated MyD88 further recruits different proteins like interleukin 1 receptor associated kinase 1 (IRAK1), interleukin 1 receptor associated kinase 4 (IRAK4), TRAF6 and TAK1, ultimately resulting in activation of NF-κb and MAPK pathways shown in Figure 13. Both pathways leads to activation of plethora of inflammatory cytokines as well as anti-apoptotic signalling resulting in cell survival (229, 241).

Figure 13: TLR signalling schematic overview. Reproduced with permission (242)
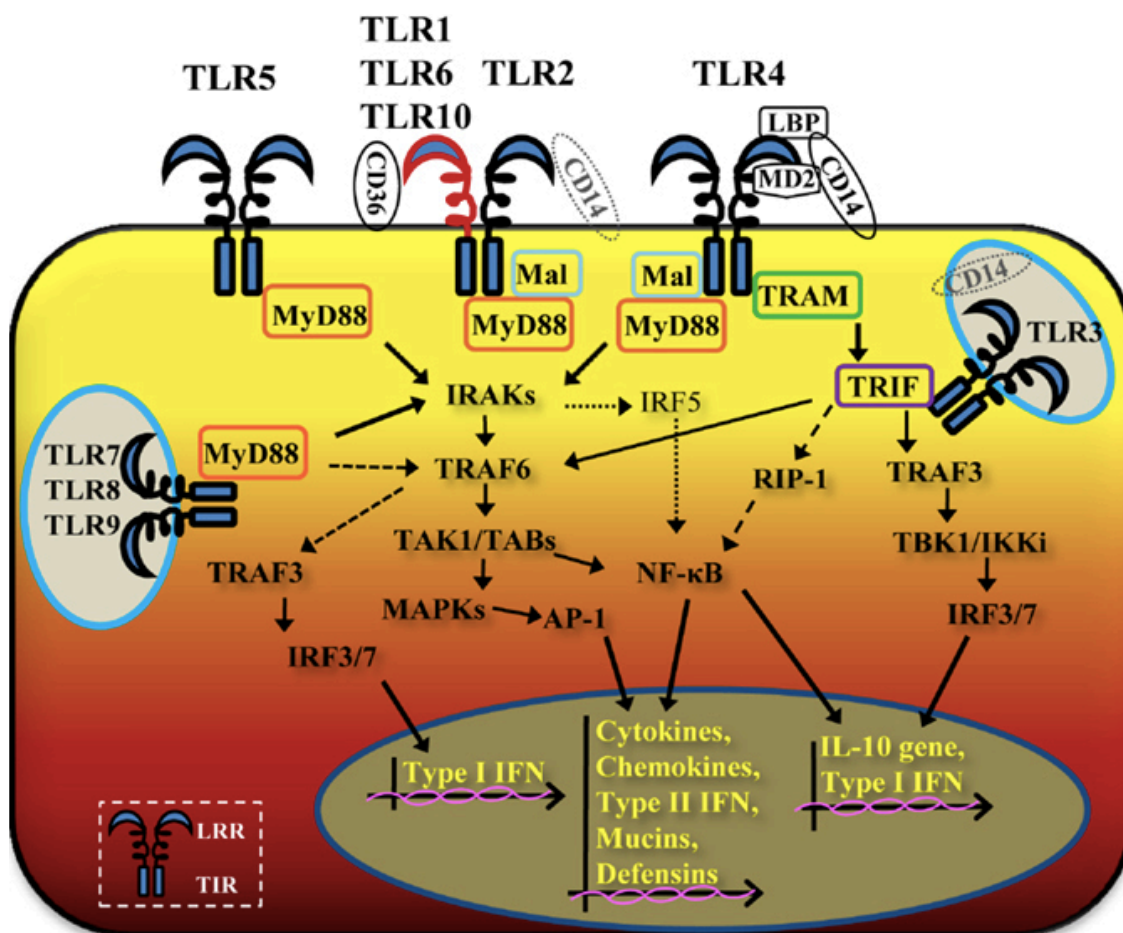
On the other hand, MyD88-independent pathway is activated through TRIF downstream of TLR3 and TLR4. It is important to note that TLR3 can directly recruit TRIF while TLR4 recruit it indirectly through TRIF related adapter molecule (TRAM) (243). TRIF can regulate TRAF6 ultimately resulting in release of type 1 interferons (244). Furthermore, TRIF can interact with RIPK1 that plays key role in the decision of cell survival or cell death (245). Deficiency of Ripk1 leads to impaired NF-κb activation. It has also been demonstrated that impaired NF-κb activation does not depend on the kinase activity of RIPK1 (246). In the presence of RIPK1, TLR3 or TLR4 activation does not lead to necroptosis until activity of FADD-Caspase 8-cFLIP is inhibited or disrupted (246, 247). This type of necroptosis can be inhibited by

pharmacological or genetic inhibition of *Ripk1* kinase activity (246). It can also be inhibited by targeting RIPK3 or MLKL activity as shown in figure 14 (57).

Figure 14. TLR3/TLR4 induced Necroptosis signalling in wild type and Ripk1 deficient cells adapted from (247)
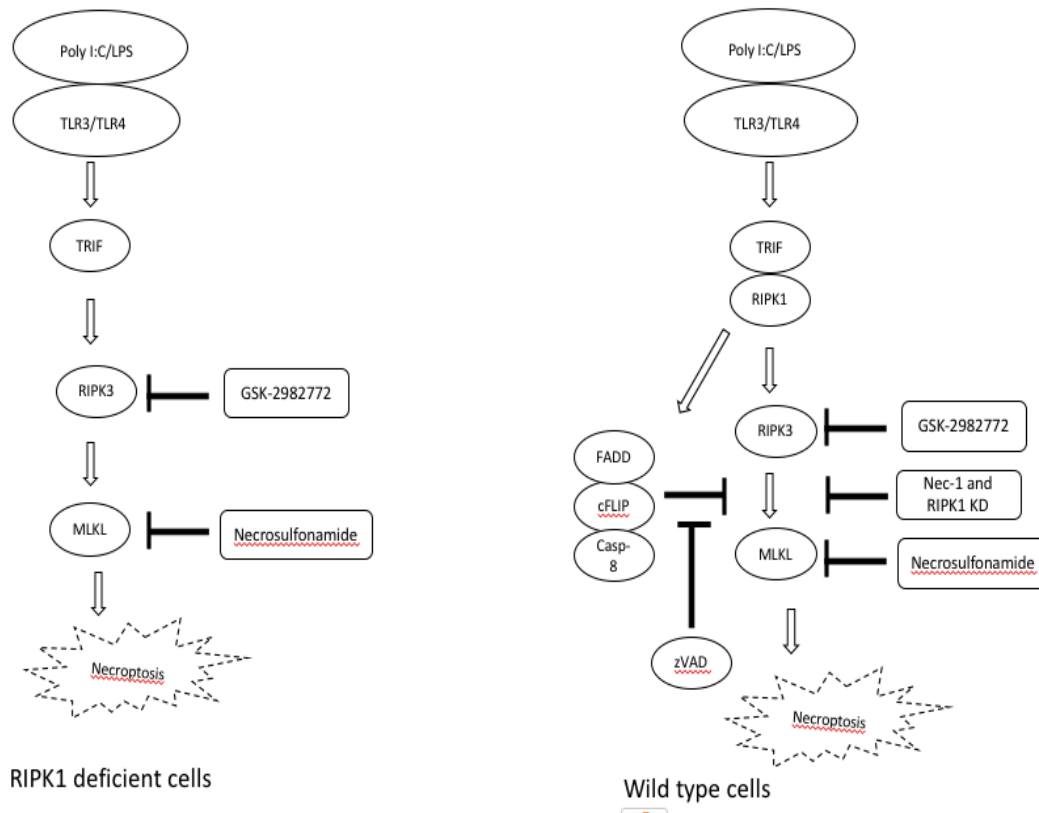
On the other hand, in the absence of RIPK1, TRIF can directly bind to RIPK3 resulting in necroptosis (247). This type of necroptosis is independent of RIPK1 and cannot be inhibited by targeting RIPK1 kinase activity (57). Recently, it has been demonstrated that Poly I:C and LPS leads to increased death of skin fibroblasts isolated from Ripk1-deficient patients. This death cannot be inhibited by Zvad treatment while RIPK3 and MLKL inhibition resulted in amelioration of cell survival demonstrating that *Ripk1*-deficiency leads to necroptosis downstream of TLR3 and TLR4 signalling (57).

Ripk1-/- mice (deficiency in all tissue), tend to die within the first 3 days of their life. Even if these mice were apparently normal without any gross lesions, histological examination revealed massive cell death in lymphoid as well as adipose tissue suggesting the key role of RIPK1 in homeostasis of these tissues (248). Further analysis revealed NF-κB defective signalling in *Ripk1* deficient cells downstream of TNFR1 signalling (248). Additionally, Ripk1 kinase dead knock in mice (*Ripk1^{KD}*) were normal suggesting the kinase independent function of RIPK1 in survival. Therefore, kinase independent function(s) of RIPK1 is or are responsible for the early mortality of *Ripk1*-/- mice (249). Unlike *Ripk1^{KD}* mice, *Ripk1* RHIM deficient mice die within the first week of life, further underlying the kinase-independent role of Ripk1 in homeostasis (250, 251). Combined inhibition of apoptosis and necroptosis through different genetic modifications like *Ripk3* and *Fadd* or *Ripk3* and caspase-8 in *Ripk1*-/- mice resulted in prolonged survival, suggesting the possible involvement of both apoptosis and necroptosis in inducing mortality in *Ripk1*-/- mice (247, 252). Furthermore, another research revealed that combined deficiency of *Trif* and *Tnfr1* in *Ripk1*-/- mice also resulted in improved survival (247). These studies strongly suggest protective role of RIPK1 in homeostasis.

Conditional depletion of *Ripk1* (*Ripk^{IEC-KO}*) in intestinal epithelium leads to stunted growth, early mortality, decreased goblet cells and Paneth cells, villus atrophy due to massive cell death in the intestine. However, additional deletion of *Tnfr1*, *Trif* or *Fadd* in *Ripk1^{IEC-KO}* resulted in amelioration in the intestinal pathology and further improved survival. Nonetheless, the additional deletion of *Fadd* resulted in RIPK3 dependent necroptosis. Besides, conditional deletion of *Ripk1* in epidermal tissue (*Ripk1^{E-KO}*) resulted in increased epidermal inflammation that can be ameliorated by additional deletion of *Tnfr1* or *Ripk3* (253, 254). In hematopoietic stem cells, *Ripk1* deficiency led to Ripk3-dependent cell death (255). Finally, we and others have demonstrated that *Ripk1^{LPC-KO}* mice specifically deficient for *Ripk1* in liver parenchymal

cells, including hepatocytes, develop normally as compared to their WT littermates (167, 256, 257). In conclusion, studies using conditional deletion of *Ripk1* demonstrated its kinase-dependent tissue specific role in homeostasis. However, RIPK1 remains dispensable for liver homeostasis. Therefore, availability of these genetically modified mice and pharmacological inhibition of RIPK1 through Nec-1 enabled scientific community to explore respectively the role of RIPK1 and its kinase activity in liver diseases.

5.5 ROLE OF RIPK1 IN LIVER DISEASES

RIPK1 regulates cell death and survival induced in response to death ligand or PAMPs (40). RIPK1 has distinct kinase dependent pro-death or kinase independent scaffolding dependent pro-survival role (199, 249). In recent years, RIPK1 is one of the most widely studied molecule in liver diseases. Previously, we have demonstrated that RIPK1 in liver parenchymal cells is dispensable for liver homeostasis (no histological abnormalities in *Ripk1^{LPC-KO}* mice). We have also demonstrated that *Ripk1* depletion in liver parenchymal cells sensitizes to ConA-induced hepatitis, while its kinase activity inhibition by Nec-1s pre-treatment or through genetic manipulation, limited ConA-induced symptoms (167). These findings suggested that RIPK1 scaffolding and its kinase activity could have distinct role in liver pathologies. Suda *et al.* also reported that inhibition of *Ripk1* through ASO pre-treatment resulted in increased liver damage in response to ConA (258). In addition, neutralization of TNF-α by pre-treatment with Etanercept (ETA), a TNF-α decoy receptor, resulted in significant decrease in ConA induced liver damage in *Ripk1^{LPC-KO}* mice, suggesting the involvement of TNF-α (167). Furthermore, RIPK1 was shown to be protective in TNF-α-induced hepatitis by stabilizing TRAF2. Primary mouse hepatocytes (PMH) derived from *Ripk1^{LPC-KO}* mice tends to die spontaneously (167). Further experiments revealed that these isolated PMH died by apoptosis and mainly through TNF-α (167, 257). RIPK1 and TRAF2 seems to be compensatory for each other as deficiency of either molecule is dispensable for liver homeostasis while combined ablation resulted in

spontaneous HCC in mice (256). In ConA-induced liver damage, neutralization of TNF-α is not fully protective in *Ripk1^{LPC-KO}* mice suggesting the involvement of other death ligands like FASL and TRAIL (167). Liver transcripts of both FASL, TRAIL and their cognate receptors were upregulated in ConA-induced hepatitis (166). Thus, we also revealed the protective function of RIPK1 in Jo2 (Anti-Fas antibody) and TRAIL+IFN gamma induced liver damage (166). In contrast, Suda *et al.* described that Ripk1 does not contribute to Jo2 induced liver damage (258). This discrepancy can be explained by the different methods employed to deplete Ripk1, as ASO depleted RIPK1 transiently and its depletion is not as efficient as in in *Ripk1^{LPC-KO}* mice.

We and others have also shown that RIPK1 also protect from LPS-induced liver injury (256, 257, 259). The injury observed in ConA-treated *Ripk1^{LPC-KO}* mice was caused by TNF-α secreted by KCs as neutralization of TNF-α or depletion of macrophages resulted in protection from LPS-induced liver injury (259). Another study showed that inhibition of RIPK1 kinase activity led to amelioration of LPS/D-Gal induced liver damage (45).

RIPK1 kinase activity also contributes in APAP induced hepatotoxicity *in vivo* and *in vitro* (134, 260-264). Thus, inhibition of *Ripk1* by ASO pre-treatment protected mice from APAP induced hepatotoxicity (134). In contrast, *Ripk1* deficiency in liver parenchymal cells did not sensibilized to APAP induced hepatotoxicity (135). These conflicting results could be due to the different modes of depletion of *Ripk1* or to RIPK1 kinase activity in liver non-parenchymal cells. Furthermore, independently of RIPK1 kinase activity, *Ripk1* deficiency plays protective role in diethyl nitrosamine (DEN) induced tumorigenesis (257). Accordingly, DEN-induced TNF-α secretion that resulted in early apoptosis in *Ripk1^{LPC-KO}* mice, hence preventing cancer cell emergence and consequently decreased liver tumorigenesis. This hypothesis is further consolidated with the evidence that additional deletion of TNFR1 in *Ripk1^{LPC-KO}* mice promoted hepatic tumorigenesis. In conclusion, this study revealed the scaffolding function of RIPK1 in

the DEN model, that increased survival of tumorigenic cells through TNF-α-induced NF-κb pathway activation (257).

Recently, patients with complete deficiency of *RIPK1* due to rare homozygous mutations have been identified. They are immunodeficient accompanied by lymphopenia, suffer from recurrent viral, bacterial and fungal infections and early onset of arthritis and inflammatory bowel disease(57, 265). Fibroblast derived from patients stimulated with death ligand TNF-α or PAMPs (LPS and Poly I:C), revealed impaired inflammatory signalling and increased cell death. All patients under investigation, died between age 3-13 years except one which was successfully treated by hematopoietic stem cell transplantation (57). This suggests the therapeutic potential of haematopoietic transplantation but necessitates further investigations.

The summary of experimental designs and conclusions targeting role of RIPK1 in various liver pathologies are shown in table 7.

Table 7. Summary of Role of Ripk1 in various liver pathologies

Hepatitis models	Experimental design	Conclusions	Reference:
APAP induced hepatotoxicity	Mice were pre-treated with Nec-1, 1 hour before injection of APAP	APAP induced hepatotoxicity results in induction of RIPK1 and Nec-1 pre-treatment ameliorated liver damage	(260)
	Primary mouse hepatocytes were pre-treated with Nec-1 prior to treatment of APAP	Nec-1 pre-treatment in cells significantly protected against APAP induced cellular damage at 24 hours post treatment but not after 48 hours.	(261)
	Mice were pre-treated with Nec-1, 30 mins before administration of APAP	APAP induced hepatotoxicity results in induction of RIPK1 and Nec-1 pre-treatment results in amelioration of APAP induced liver damage	(262)
	Mice were pre-treated with Nec-1, 15 mins before administration of APAP	Nec-1 treatment results in amelioration of liver damage	(263)
	APAP was administered in mice in which Ripk1 was knocked down by anti-sense oligonucleotides pre-treatment. And mice and PMH were also pre-treated with Nec-1 before APAP treatment.	RIPK1 is protective and Nec-1 also resulted in amelioration in APAP induced cellular necrosis both in vivo and in vitro.	(134)
	Mice were pre-treated with Nec-1 and Nec-1s before APAP administration	Both Nec-1 and Nec-1s are protective in APAP induced liver damage.	(264)
	Ripk1LPC-KO mice and their littermates were administered with APAP	RIPK1 deficiency in liver parenchymal cells neither protected nor sensitized to APAP induced necrotic cell death	(135)
Con A induced liver damage	Mice were pre-treated with Nec-1 prior to administration of ConA	Nec-1 results in the amelioration of liver damage.	(266)
	C57BL/6 mice were exposed to Con A and Nec-1	Con A treatment in elevated expression of Ripk1 and Nec-1 resulted in amelioration of ConA induced hepatocyte damage and decrease in inflammatory markers.	(267)
	Mice were pre-treated with Nec-1 prior to administration of ConA	Nec-1 treatment leads to less hepatocyte damage and decreased inflammation	(268)

		Mice were pre-treated with Nec-1 prior to administration of ConA	Nec-1 treatment resulted in increase in liver damage which was also confirmed by Nec-1s.	(264)
		Anti-sense oligonucleotides induced Ripk1 knockdown mice were treated with ConA	Depletion of Ripk1 resulted in elevated liver damage which is caspase dependent	(258)
		RIPK1 kinase dead knock-in mice (Ripk1K45A) and Ripk1LPC-KO mice were treated with ConA and systematically compared to sex and age matched littermates. Nec-1s was also used as an inhibitor of kinase activity of RIPK1.	Depletion of chemical inhibition of kinase activity of RIPK1 resulted in protection while depletion of Ripk1 specifically in liver parenchymal cells resulted in elevated liver damage which is caspase dependent	(167)
LPS induced liver damage		Anti-sense oligonucleotide induced Ripk1 knockdown mice were treated with LPS	Ripk1 knockdown resulted in massive increase in caspase dependent cell death.	(258)
		Ripk1LPC-KO mice were treated with LPS and systematically compared to sex and age matched littermates. Mice were also pre-treated with ETA (TNF-α decoy receptor) and macrophages were depleted by administration of liposome chlodronate pre-administration of LPS.	Depletion of Ripk1 specifically in liver parenchymal cells resulted in elevated liver damage which is caspase dependent and carried out through TNF-α produced from macrophages	(259)
		Ripk1LPC-KO mice were treated with LPS and systematically compared to sex and age matched littermates	RIPK1 plays protective role in LPS induced hepatocyte damage and Ripk1 deficient hepatocytes has decreased NFκb signaling.	(257)
		Ripk1LPC-KO mice were treated with LPS and systematically compared to sex and age matched littermates	RIPK1 plays protective role in LPS induced hepatocyte damage	(256)
		Mice were pre-treated with Nec-1 prior to administration of LPS/D-Gal.	Nec-1 ameliorates LPS/D-Gal induced hepatocyte damage.	(45)
DEN induced HCC		Ripk1LPC-KO and Ripk1 kinase dead mice were treated with DEN	Depletion of Ripk1 specifically in liver parenchymal cells sensitizes to early DEN induced liver damage. While at later stage, it showed reduced liver tumorigenesis. While kinase activity did not showed amelioration in DEN induced HCC.	(257)

II. OBJECTIVES

OBJECTIVES

Hepatocyte cell death plays important role in liver pathologies. Persistent chronic liver damage can further progress into liver cirrhosis, fibrosis and HCC, the 3rd leading cause of cancer related deaths. Cell death is associated with elevated expression or secretion of death ligands by the immune cells. Death ligands, mainly TNF-α, FASL and TRAIL, induce cell death by binding their cognate receptors TNFR1, FAS and TRAILRs, respectively. Downstream of these death ligand receptors, RIPK1 plays key role in decision of cell's fate to live or die. RIPK1 has distinct kinase-dependent pro-death and kinase-independent pro-survival functions. Previously, we found that RIPK1 depletion, specifically in liver parenchymal cells, sensitizes mice to immune mediated hepatitis induced by ConA. Additionally, we discovered that pharmacological or genetic inhibition of RIPK1 kinase activity limits liver damage induced by ConA. Thus, RIPK1 expressed in hepatocytes provides a protective role for the cell in the presence of some death ligands, such as TNF-α, FASL and TRAIL combined to IFN gamma. This protection property is also effective in a pathological context with abnormal amounts of bacterial PAMPs. Our investigations suggested that these PAMPs stimulates Kupffer cells for the production of TNF-α, a death ligand against which hepatocytes are resistant, partly because of RIPK1. The aim of my thesis work was to further extend our understanding on the role of RIPK1 in liver diseases by addressing the following four aspects:

- Explore the role of RIPK1 in fulminant viral hepatitis.

- Elucidate the role of RIPK1 in APAP- and CCl_4-induced acute liver injury.

- Investigate the role of RIPK1 in HFHCD induced NASH.

- Determine the efficacy of dietary intervention in reducing NASH-induced fibrosis.

III. RESULTS

Article 1: Depletion of RIPK1 in hepatocytes exacerbates liver damage in fulminant viral hepatitis.

Background and aims

Fulminant viral hepatitis is a world-wide syndrome caused by hepatotropic (HAV, HBC, HCV, HEV) and non-hepatotropic viruses (cytomegalovirus and herpes simplex virus). This affection is characterized by massive liver damage, hepatic encephalopathy and multiple organ failure. Since the protein kinase RIPK1 is known to play a crucial function at the crossroad of stress-induced signalling pathways that affects cell's decision to live or die, its role in hepatocytes during fulminant viral hepatitis was investigated.

Methods

To, partially or totally, experimentally reproduce fulminant viral hepatitis, we used two murine models respectively based on the administration of poly I:C, a synthetic analog of double-stranded RNA mimicking viral pathogen-associated molecular pattern, or of Murine Hepatitis Virus type 3 (MHV3). Mice deficient for RIPK1, specifically in liver parenchymal cells ($Ripk1^{LPC-KO}$) and their wild-type littermates ($Ripk1^{fl/fl}$), were thus challenged by either the poly I:C or(MHV3) . Liver damage was assessed by measurement of plasma level of transaminases (ALT and AST) and by liver histology and immune histochemistry (cleaved caspase-3). Expression of cytokines was measured at the transcript level by RT-QPCR in liver tissue and at protein level by LEGENDplex™ in plasma. To explore the involvement of TNF-α and macrophages, mice were respectively pre-treated with ETA, a decoy receptor of TNF-α, or clodronate-loaded liposomes which specifically deplete, macrophages, prior poly I:C administration.

Results

As evidenced by plasma concertation of both ALT and AST, liver damage was more pronounced in *Ripk1*^{LPC-KO} mice 48 hours after their inoculation with MHV3, difference that disappeared at later time (72 h post-inoculation). However, infection levels and plasma concentrations of pro-inflammatory cytokines, such as TNF-α and CCL2, did not differ between the 2 mouse genotypes. Furthermore, administration of poly I:C triggered an increase in systemic transaminases only in *Ripk1*^{LPC-KO} mice, reflecting liver damage through induced apoptosis, as illustrated by cleaved-caspase 3 labelling of liver tissue sections. Neutralization of TNF-α or prior depletion of macrophages protected *Ripk1*^{LPC-KO} mice from poly I:C induced hepatitis as evidenced by the maintenance of the basal level of plasma transaminases (ALT and AST). Moreover, poly I:C never induced direct hepatocyte death in primary culture, whatever the murine genotype, while it always stimulated an antiviral response.

Conclusion

Our results demonstrated that RIPK1 protects hepatocytes from TNF-α secreted from macrophages during viral induced fulminant hepatitis. These data emphasize the potential worsening risks of an HBV infection in people with polymorphism or homozygous amorphic mutations already described for the RIPK1 gene.

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Depletion of RIPK1 in hepatocytes exacerbates liver damage in fulminant viral hepatitis

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Abstract
The protein kinase RIPK1 plays a crucial role at the crossroad of stress-induced signaling pathways that affects cell's decision to live or die. The present study aimed to define the role of RIPK1 in hepatocytes during fulminant viral hepatitis, a worldwide syndrome mainly observed in hepatitis B virus (HBV) infected patients. Mice deficient for RIPK1, specifically in liver parenchymal cells (*Ripk1*[LPC-KO]) and their wild-type littermates (*Ripk1*^{fl/fl}), were challenged by either the murine hepatitis virus type 3 (MHV3) or poly I:C, a synthetic analog of double-stranded RNA mimicking viral pathogen-associated molecular pattern. *Ripk1*[LPC-KO] mice developed more severe symptoms at early stage of the MHV3-induced fulminant hepatitis. Similarly, administration of poly I:C only triggered increase of systemic transaminases in *Ripk1*[LPC-KO] mice, reflecting liver damage through induced apoptosis as illustrated by cleaved-caspase 3 labeling of liver tissue sections. Neutralization of TNF-α or prior depletion of macrophages were able to prevent the appearance of apoptosis of hepatocytes in poly I:C-challenged *Ripk1*[LPC-KO] mice. Moreover, poly I:C never induced direct hepatocyte death in primary culture whatever the murine genotype, while it always stimulated an anti-viral response. Our investigations demonstrated that RIPK1 protects hepatocytes from TNF-α secreted from macrophages during viral induced fulminant hepatitis. These data emphasize the potential worsening risks of an HBV infection in people with polymorphism or homozygous amorphic mutations already described for the *RIPK1* gene.

Introduction

Fulminant viral hepatitis is a worldwide syndrome, which requires immediate intensive care, and is associated with massive hepatocyte death, hepatic encephalopathy, and multiple organ failure[1]. Even if non-hepatitis viruses, such as the herpes simplex virus (HSV) or the cytomegalovirus (CMV) are occasional etiological factors in immunocompromised patients, most fulminant hepatitis with an identifiable viral cause originates from infection with hepatotropic virus, mainly the hepatitis B virus (HBV) and more rarely the hepatitis A, C, or E viruses (HAV, HCV, HEV)[2,3]. Orthotopic liver transplantation remains the leading life-saving option for patients with fulminant hepatitis[4,5]. Nowadays, mouse models of murine hepatitis virus type 3 (MHV3) infection or polyinosine-polycytidylic acid (poly I:C) injection emerge as effective tools to investigate the underlying mechanisms in pathogenesis and to test novel therapeutic strategies in this disease[6–8].

Indeed, the more physiologically relevant in vivo model to study fulminant viral hepatitis is MHV3 infection. This coronavirus is a single stranded, positive sense RNA virus. It is highly pathogenic and could cause death in 3–5 days depending on age, route of infection, viral doses, murine strain, and on the immune status of the animal[9]. Different

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physiopathological issues take place during the course of the disease. In the liver, the cell tropism of MHV3 results in the infection of resident macrophages (Kuppfer cells), liver sinusoidal endothelial cells, hepatic stellate cells, and hepatocytes[10]. Host-pathogen interactions cause release of high levels of inflammatory mediators while repressing the production of immunosuppressive factors[11–14]. Concomitantly, activated macrophages and sinusoidal endothelial cells secrete the prothombinase fibrinogen-like protein 2 (FGL2), initiating fibrin matrix formation. The induced coagulopathy disrupt blood supply leading ultimately to liver necrosis[1].

Regarding poly I:C, it is a synthetic analog of double-stranded RNA. This pathogen associated molecular pattern (PAMP) interacts with different cellular pattern recognition receptors (PRR), including the Toll like receptor 3 (TLR3), the cytosolic protein kinase RNA-activated (PKR) and the melanoma differentiation-associated protein 5 (MDA5)[15,16]. Upon extracellular double-stranded RNA recognition, TLR3 recruits the cytosolic TIR-domain-containing adapter-inducing interferon-β (TRIF). Through kinases, this adaptor protein induces the nuclear translocation of the interferon regulatory factor 3 (IRF3) which drives the expression of anti-viral type I interferons (IFNs). In another signal transduction cascade, TRIF engages the receptor interacting protein kinase-1 (RIPK1) to promote downstream activation of NF-κB, eliciting the production of pro-inflammatory cytokines, such as TNF-α, IL-6, IL-1β, and chemokines, mainly by Kuppfer cells and natural killer (NK) cells[17,18]. As for Poly I:C, it causes liver damage in mice only if pre-treated with D-galactosamine (D-GalN). This amino sugar specifically blocks the transcription in hepatocytes[19]. Thus, the TNF-α-induced NF-κB pathway cannot lead to the synthesis of the anti-apoptotic proteins. Hepatocytes then die by TNF-α activated apoptosis[8,20].
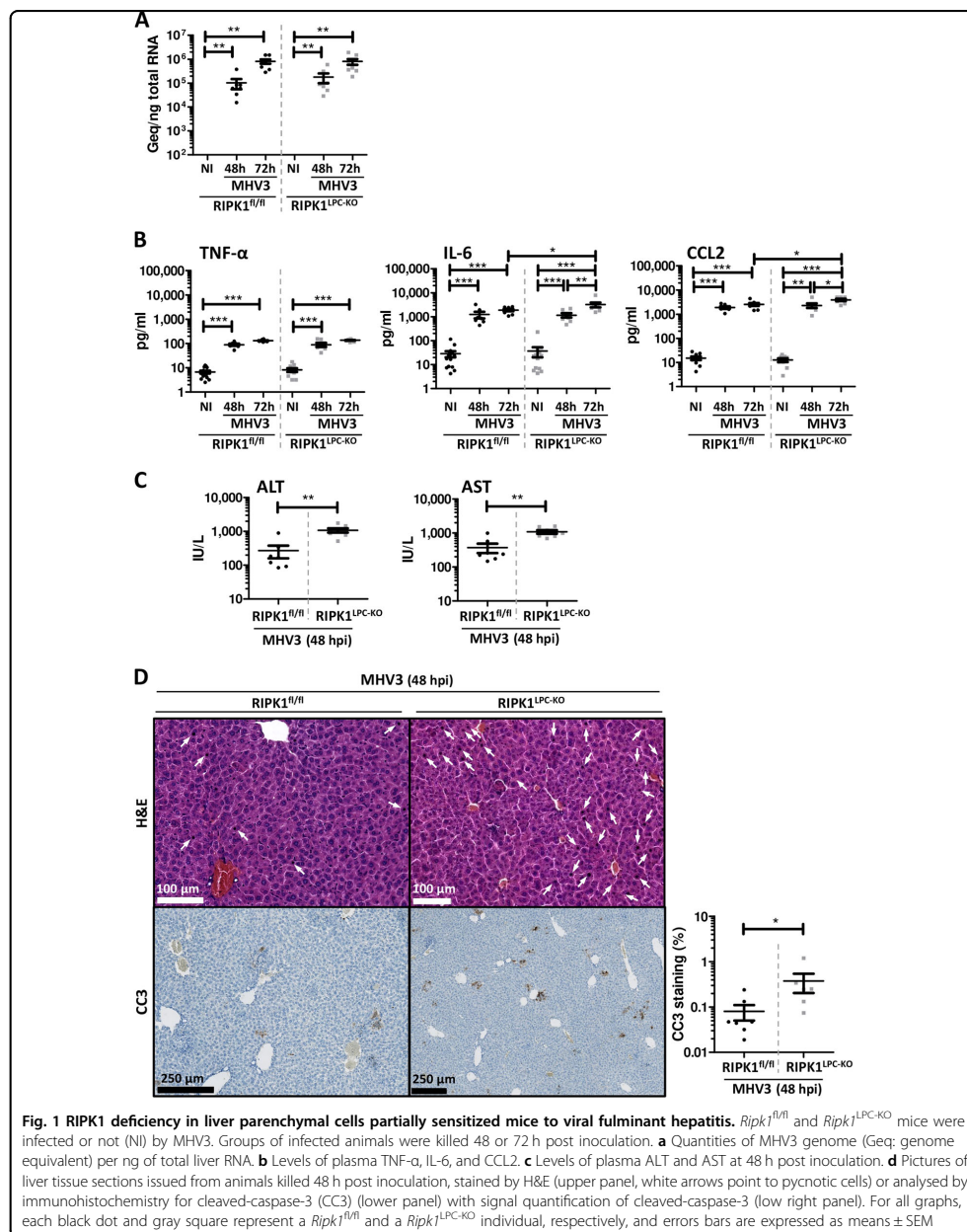
TNF-α is a key cytokine involved in both acute and chronic liver diseases, like fulminant hepatic failure, alcohol-induced hepatitis, viral hepatitis, metabolic toxicity, drug-induced liver injury and autoimmune hepatitis. This death factor is recognized by the receptors TNFR1 and TNFR2, but most of its biological activity depends on TNFR1[21]. Downstream of TNFR1, RIPK1 serves as a signaling node to play key roles in regulating cell survival, caspase-dependent apoptosis and RIPK3/mixed lineage domain-like pseudokinase (MLKL)-dependent necroptosis[22]. In previous works, we demonstrated that RIPK1 expressed in liver parenchymal cells provides a protective function during murine hepatitis-induced either directly by TNF-α[23] or by bacterial pathogen associated molecular patterns (e.g. Lipopolysaccharide or unmethylated CpG oligodeoxynucleotide) which activate Kuppfer cells for TNF-α production[24]. In the present study, we now investigate the unknown role of RIPK1 during the specific circumstances of fulminant viral hepatitis.

Results

RIPK1 deficiency sensitized mice to MHV3-induced liver damage

We first investigated the potential functions that RIPK1 could play in liver parenchymal cells during fulminant viral hepatitis by taking advantage of a physiological murine model based on MHV3 infection. A mouse strain deficient for RIPK1 specifically in liver parenchymal cells (*Ripk1*[LPC-KO]) and their controls (*Ripk1*^{fl/fl} littermates) were inoculated with low quantities of viruses. Beforehand, we checked that the introduced genetic modification did not affect the health status of the mouse liver in basal conditions. Indeed, normal transaminase levels were measured in the plasma of these *Ripk1*[LPC-KO] animals, as for their *Ripk1*^{fl/fl} littermates (87.4 ± 11.1 versus 69.7 ± 13.7 IU/L for ALT, respectively). Similarly, histological observations of their liver did not reveal any anomaly. Animals from both groups were included in an experimental protocol for MHV3-induced fulminant viral hepatitis. The infection levels of each individual were followed by assessing the amount of viral genomes present in their liver at 48 and 72 h post inoculation (hpi) (Fig. 1a). No significant differences were detected between *Ripk1*[LPC-KO] and *Ripk1*^{fl/fl} mice, showing that RIPK1 deficiency in parenchymal cells of the liver did not alter the MHV3 infection and replication. As pro-inflammatory cytokines play a key role in the development of fulminant viral hepatitis, we investigated their regulations in infected mice. Infection triggered similar cytokine storms in both genotypes as illustrated by the equivalent large quantities found in the bloodstream of *Ripk1*[LPC-KO] and *Ripk1*^{fl/fl} mice, such as for TNF-α, IL-6, and CCL2 (Fig. 1b). These findings mainly followed the inductions observed at the transcript levels (Supplementary Fig S1B). However, the systemic concentrations of some pro-inflammatory cytokines (IL-6 and CCL2), but not all (TNF-α, IL-1α, and IL-1β), were slightly over-induced in *Ripk1*[LPC-KO] mice at late stage of infection (72 hpi). Besides, the fulminant hepatitis severity was more pronounced in *Ripk1*[LPC-KO] mice at 48 hpi. Thus, higher liver damage in *Ripk1*[LPC-KO] mice were evidenced by elevated plasma AST/ALT transaminases (Fig. 1c). This was confirmed by the histological examination of livers. Accordingly, H&E staining revealed the presence of more pyknosis, a phase preceding cell death, in tissue sections from *Ripk1*[LPC-KO] compared to those of *RipK1*^{fl/fl} littermates (Fig. 1d, upper panels). Furthermore, *Ripk1*[LPC-KO] liver sections also revealed higher levels of cleaved caspase-3 (Fig. 1d, lower panels). In fact, as MHV3 continued to replicate in all animals, the hepatitis progressed and all differences observed at 48 hpi disappeared at 72 hpi (Supplementary Fig S2A and S2B).

Fig. 1 RIPK1 deficiency in liver parenchymal cells partially sensitized mice to viral fulminant hepatitis. *Ripk1*^{fl/fl} and *Ripk1*[LPC-KO] mice were infected or not (NI) by MHV3. Groups of infected animals were killed 48 or 72 h post inoculation. **a** Quantities of MHV3 genome (Geq: genome equivalent) per ng of total liver RNA. **b** Levels of plasma TNF-α, IL-6, and CCL2. **c** Levels of plasma ALT and AST at 48 h post inoculation. **d** Pictures of liver tissue sections issued from animals killed 48 h post inoculation, stained by H&E (upper panel, white arrows point to pycnotic cells) or analysed by immunohistochemistry for cleaved-caspase-3 (CC3) (lower panel) with signal quantification of cleaved-caspase-3 (low right panel). For all graphs, each black dot and gray square represent a *Ripk1*^{fl/fl} and a *Ripk1*[LPC-KO] individual, respectively, and errors bars are expressed as means ± SEM

These findings suggest that RIPK1 plays a partial protective role at early stages of fulminant viral hepatitis.

Deficiency of RIPK1 in liver parenchymal cells sensitized mice to poly I:C administration

To pursue investigations on the role of RIPK1 during viral hepatitis, we exposed $Ripk1^{LPC-KO}$ and $Ripk1^{fl/fl}$ mice to a molecular pattern associated with viral infection, the poly I:C. Adult $Ripk1^{LPC-KO}$ and $Ripk1^{fl/fl}$ animals were subjected to a unique low dose of poly I:C (1.5 µg/g of body weight) by intravenous injection. The synthetic double-stranded RNA triggered an antiviral response in the liver of both $Ripk1^{LPC-KO}$ and $Ripk1^{fl/fl}$ mice as demonstrated by the transcriptomic induction of some interferon-inducible antiviral effectors: PKR, the GTP-binding protein Mx1 and the 2′−5′-oligoadenylate synthetase (OAS1c) (Supplementary Fig S3A). To compare the inflammatory response induced in $Ripk1^{LPC-KO}$ and $Ripk1^{fl/fl}$ mice challenged with poly I:C, we measured the systemic concentrations of a panel of pro-inflammatory cytokines. Whatever the tested murine genotype, administration of synthetic viral nucleic acid pattern resulted in significant increased concentrations of TNF-α, IL-6 and CCL2 (Fig. 2a). Exacerbated inductions were detected in the $Ripk1^{LPC-KO}$ strain for the monocyte chemoattractant CCL2. This enhanced upregulation in $Ripk1^{LPC-KO}$ mice was found at the transcript level of these 3 cytokines (Supplementary Fig S3B). Regarding plasma transaminases, while both AST and ALT levels remained at physiological concentrations in $RipK1^{fl/fl}$ mice 8 h post injection, a massive release of transaminases was observed in the blood of poly I:C-treated $Ripk1^{LPC-KO}$ mice (Fig. 2b). On histological examination, liver sections from $Ripk1^{LPC-KO}$ revealed more and wider necrotic foci through H&E staining in contrast to their $Ripk1^{fl/fl}$ littermates (Fig. 2c, upper panels). Accordingly, some apoptotic cells appeared only in $Ripk1^{LPC-KO}$ liver tissues as revealed by cleaved caspase-3 labelling (Fig. 2c, lower panels). These findings suggested that the presence of RIPK1 in hepatocytes helps to protect parenchymal cells from the host immune response triggered by the sensing of viral nucleic acid patterns.

Neutralization of TNF-α protected $Ripk1^{LPC-KO}$ mice from poly I:C-induced liver damage

As highlighted above, an inflammatory response was established in murine individuals exposed to poly I:C. Among released cytokines, blood concentrations of TNF-α were increased by more than five times in $Ripk1^{LPC-KO}$ mice as in their $Ripk1^{fl/fl}$ littermates (Fig. 2a). In our previous reported studies dealing with non-viral origin hepatitis induced in $Ripk1^{LPC-KO}$ mice, death of RIPK1-deficient hepatocytes were directly attributed to high doses of this cytokine[2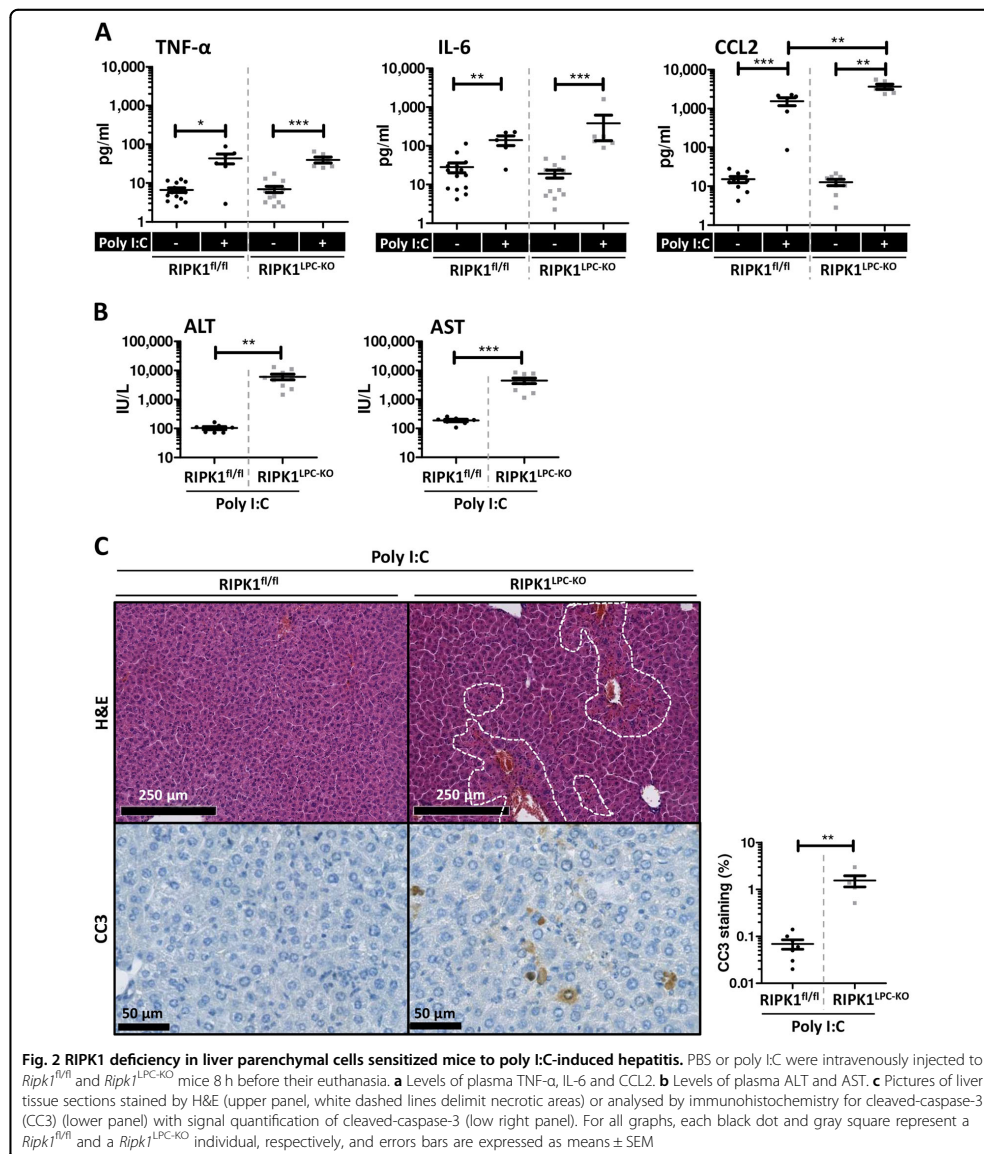3,24]. To test this mechanistic hypothesis in the hepatitis model induced by a viral pattern, we administered ETA, a decoy receptor of TNF-α, 1 h prior poly I:C injection. Neutralization of TNF-α proved to be sufficient to prevent the occurrence of liver damage in $Ripk1^{LPC-KO}$ mice. Indeed, plasma AST/ALT levels remained physiological (Fig. 3a) and little or no necrotic foci or apoptotic cells were distinguished in $Ripk1^{LPC-KO}$ liver sections (Fig. 3b). Yet, all the poly I:C injections were successful as shown by PKR, Mx1, and OAS1c mRNA inductions (Supplementary Fig S3C). These data demonstrated that TNF-α was responsible for poly I:C-induced liver damage in $Ripk1^{LPC-KO}$ mice.

Depletion of Kupffer cells reduced poly I:C-induced hepatitis in $Ripk1^{LPC-KO}$ mice

At the time of poly I:C exposure, the liver-resident macrophages (Kupffer cells), which express at their membranes the related pattern recognition receptor TLR3, could be one of the sources of the produced TNF-α. This prompted us to specifically deplete the liver of $Ripk1^{LPC-KO}$ mice from their Kupffer cells by 2 consecutive intraperitoneal injections of clodronate-loaded liposomes (Lip-Cl₂MBP) prior to poly I:C administration. F4/80 staining of liver sections confirmed the effectiveness of Kupffer cell depletion (Fig. 4a). Besides, increased expression of hepatic PKR transcripts confirmed that each enrolled mouse was properly challenged by the poly I:C (Supplementary Fig S3D). The $Ripk1^{LPC-KO}$ mice lacking Kupffer cells became resistant to poly I:C-induced hepatitis as evidenced by diminished plasma AST/ALT levels (Fig. 4b). Likewise, the livers of these animals showed neither necrotic foci nor cleaved caspase-3 stained areas (Fig. 4c). Macrophages were therefore involved in liver damage observed in $Ripk1^{LPC-KO}$ mice challenged with poly I:C.

Poly I:C was unable to directly induce the death of RIPK1-deficient hepatocytes

To investigate the direct impact that poly I:C could have on RIPK1-deficient hepatocytes, primary cultures issued from either $Ripk1^{LPC-KO}$ or $Ripk1^{fl/fl}$ mice were prepared to be subjected to increasing concentrations of the synthetic double-stranded RNA. In contrast to their WT counterparts, primary cultures of RIPK1-deficient hepatocytes underwent substantial spontaneous death, as previously reported[23–27] (Fig. 5a). This death occurred by apoptosis since it was completely prevented by a pan-caspase inhibitor (z-VAD-fmk) (data not shown)[27]. Death in RIPK1-deficient hepatocyte primary cultures could be largely avoided by adding ETA to culture medium (Fig. 5a)[27]. This demonstrated that their death originated from sensing autocrine TNF-α. The limited remaining observed death in ETA-treated RIPK1-deficient hepatocyte primary cultures could probably be attributed in part

Fig. 2 RIPK1 deficiency in liver parenchymal cells sensitized mice to poly I:C-induced hepatitis. PBS or poly I:C were intravenously injected to *Ripk1*^{fl/fl} and *Ripk1*^{LPC-KO} mice 8 h before their euthanasia. **a** Levels of plasma TNF-α, IL-6 and CCL2. **b** Levels of plasma ALT and AST. **c** Pictures of liver tissue sections stained by H&E (upper panel, white dashed lines delimit necrotic areas) or analysed by immunohistochemistry for cleaved-caspase-3 (CC3) (lower panel) with signal quantification of cleaved-caspase-3 (low right panel). For all graphs, each black dot and gray square represent a *Ripk1*^{fl/fl} and a *Ripk1*^{LPC-KO} individual, respectively, and errors bars are expressed as means ± SEM

to cells already engaged in TNF-α signaling before ETA addition. When ETA was added to the culture medium to avoid spontaneous death of RIPK1-deficient hepatocytes, poly I:C, even at high doses (20 μg/mL), never induced the death of hepatocytes regardless of their genotype (Fig. 5b, upper panel). Experiments conducted in parallel on same batches of primary hepatocytes, but in absence of ETA, showed that addition of poly I:C, whatever the tested dose, did not cause any additional death (Fig. 5b, lower panel). Thus, in these conditions, death of RIPK1-deficient
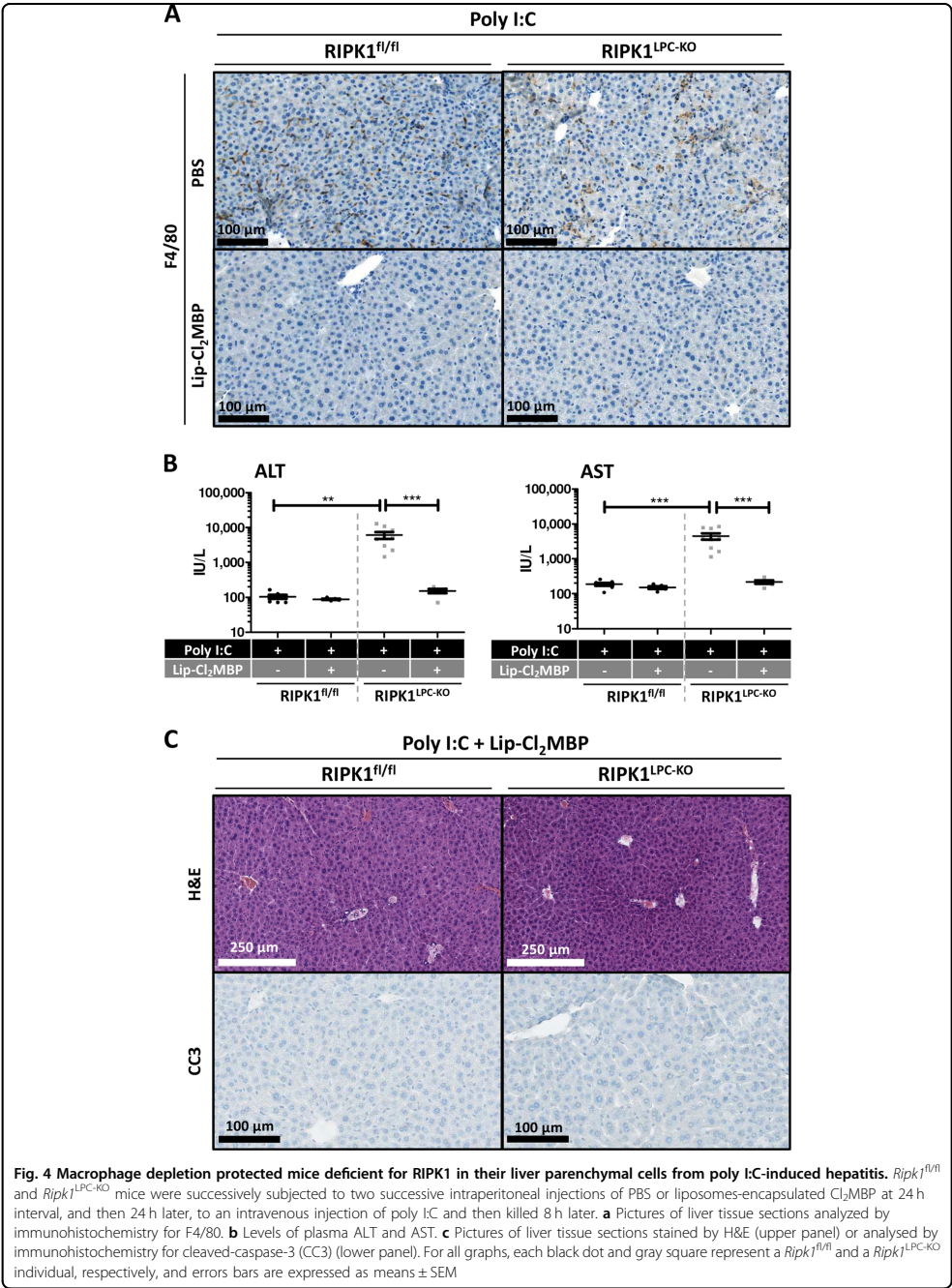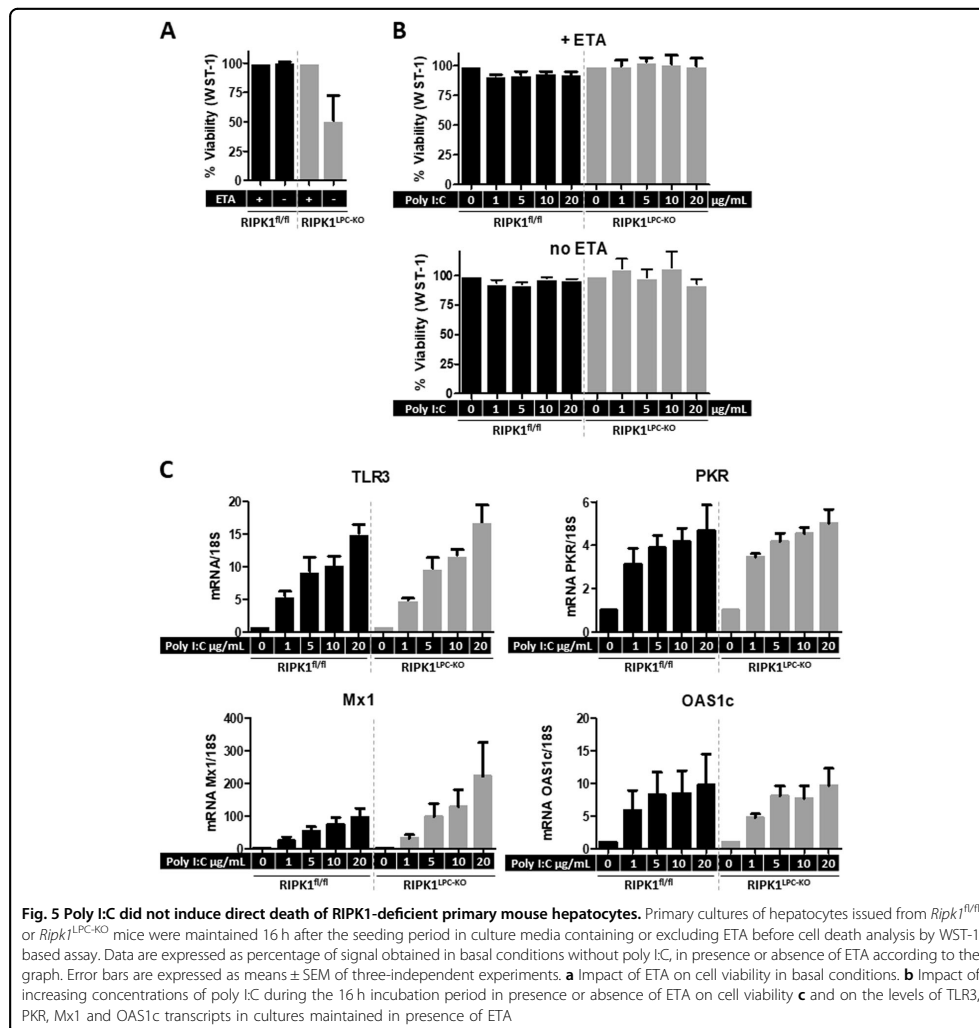
Fig. 3 The sensitivity to poly I:C of mice deficient for RIPK1 in their liver parenchymal cells was dependent on the TNF-α cytokine. *Ripk1*^LPC-KO mice underwent successively a first intraperitoneal injection of PBS or ETA, then 1 h after a second intravenous injection of poly I:C, and then killed 8 h later. **a** Levels of plasma ALT and AST. **b** Pictures of liver tissue sections stained by H&E (upper panel, white dashed lines delimit necrotic areas) or analysed by immunohistochemistry for cleaved-caspase-3 (CC3) (lower panel) with signal quantification of cleaved-caspase-3 (low right panel). For all graphs, each gray square represents a *Ripk1*^LPC-KO individual, and errors bars are expressed as means ± SEM

hepatocytes induced by endogenous TNF-α was not potentiated by poly I:C. For all these experiments, the efficacy of poly I:C signaling on primary mouse hepatocytes was checked by measuring the transcript levels of genes typically induced (TLR3, PKR, Mx1, and OAS1c). Dose-dependent upregulations were consistently observed for all tested genes, both in control and deficient hepatocytes (Fig. 5c). These findings further strengthened our notion of involvement of TNF-α, secreted by Kupffer cells, in poly I:C-induced liver damage in *Ripk1*^LPC-KO mice.

Discussion

Hepatocytes express the cytosolic kinase RIPK1 which has been described to be at the crossroad of signal transduction pathways that regulate cell fate. Indeed, RIPK1 helps to promote cell survival or switches cell to death by caspase-dependent apoptosis or by MLKL-dependent necroptosis[18]. During fulminant viral hepatitis, a cytokine burst arises, resulting in an enrichment of the hepatic microenvironment in death factors such as TNF-α, Fas ligand (FasL) or TRAIL. Once bound to their cognate receptors expressed on hepatocyte surface, the induced intracellular signal must involve RIPK1 which should impact the disease outcome, systematically characterized by massive hepatocyte death. To investigate the role of RIPK1 in fulminant viral hepatitis, mice with either normal hepatocytes (*Ripk1*^fl/fl) or defective hepatocytes for this kinase (*Ripk1*^LPC-KO) were inoculated with the MHV3. Whatever the murine genotype, all animals were equally susceptible to MHV3 infection as illustrated by the similar amounts of replicative genomes detected in their livers at identical time points. Most of tested clinical and biochemical parameters of fulminant hepatitis were

Fig. 4 Macrophage depletion protected mice deficient for RIPK1 in their liver parenchymal cells from poly I:C-induced hepatitis. *Ripk1*^{fl/fl} and *Ripk1*[LPC-KO] mice were successively subjected to two successive intraperitoneal injections of PBS or liposomes-encapsulated Cl_2MBP at 24 h interval, and then 24 h later, to an intravenous injection of poly I:C and then killed 8 h later. **a** Pictures of liver tissue sections analyzed by immunohistochemistry for F4/80. **b** Levels of plasma ALT and AST. **c** Pictures of liver tissue sections stained by H&E (upper panel) or analysed by immunohistochemistry for cleaved-caspase-3 (CC3) (lower panel). For all graphs, each black dot and gray square represent a *Ripk1*^{fl/fl} and a *Ripk1*[LPC-KO] individual, respectively, and errors bars are expressed as means ± SEM

Fig. 5 Poly I:C did not induce direct death of RIPK1-deficient primary mouse hepatocytes. Primary cultures of hepatocytes issued from *Ripk1*^fl/fl or *Ripk1*^LPC-KO mice were maintained 16 h after the seeding period in culture media containing or excluding ETA before cell death analysis by WST-1 based assay. Data are expressed as percentage of signal obtained in basal conditions without poly I:C, in presence or absence of ETA according to the graph. Error bars are expressed as means ± SEM of three-independent experiments. **a** Impact of ETA on cell viability in basal conditions. **b** Impact of increasing concentrations of poly I:C during the 16 h incubation period in presence or absence of ETA on cell viability **c** and on the levels of TLR3, PKR, Mx1 and OAS1c transcripts in cultures maintained in presence of ETA

similar between both infected mice cohorts, especially at the final stage of the infection. In particular, no difference was distinguished between the two groups of animals for parameters that influence the induction of the pro-thrombinase FGL2 expression. This pro-coagulant factor, which is at the origin of hemostasis alterations and coagulation, takes part in the pathogenicity of the MHV3 infection. Indeed, antibodies against FGL2[28] or its genetic invalidation[29] have been described to significantly

decrease the severity of MHV3-induced hepatitis. Its production is known to be triggered by a viral factor (nucleocapsid)[30] and by both cytokines TNF-α and IFNγ[31]. The lack of RIPK1 in liver parenchymal cells never altered levels of viral replication and that of the induced plasma concentrations of the above-mentioned cytokines (Fig. 1a, b and Supplementary Fig S1C), leading to similar FGL2 mRNA inductions (Supplementary Fig S1B). However, even if all animals succumbed at day 4

post-inoculation, *Ripk1*[LPC-KO] mice showed more severe signs of liver damage (plasma transaminases, apoptotic hepatocytes) at an earlier stage of infection. Although the host defense system is insufficient to protect C57BL/6 mice from fatal MHV3 fulminant hepatitis, RIPK1 appeared to partly contribute to some resistance underlying the protective function played by RIPK1 in hepatocytes. The observed partial worsening of symptoms in MHV3-infected *Ripk1*[LPC-KO] mice most probably resulted at least from their described increased sensitivity for both TNF-α and FasL[23,25,26]. Indeed, these death factors directly contribute to the death of hepatocytes and thus to pathogenesis in MHV3 fulminant hepatitis. Accordingly, significant resistance has been described for animals deficient for TNF-α or TNFR1 when infected by MHV3[32,33]. Similarly, Kuppfer cells displays more FasL at their surface in a MHV3-infected mouse, while hepatocytes also overexpress the cognate receptor (Fas). In addition, ex vivo experiments showed that a neutralizing antibody directed against FasL prevents the death of infected hepatocytes by liver natural killer (NK) cells[34].

Both mouse strains were also enrolled in a surrogate model based on injection of synthetic double-stranded RNA (poly I:C) mimicking replicative forms of viral genomes. Usually, to elicit a TLR3-regulated fulminant viral hepatitis, poly I:C should be used in combination with D-galactosamine (D-GalN)[8], a liver-specific transcriptional inhibitor[19]. However, a simple injection of poly I:C was sufficient to induce hepatitis in *Ripk1*[LPC-KO] mice, without affecting the control littermates (*Ripk1*[fl-fl]). Even if poly I:C stimulated an antiviral innate response in primary hepatocytes issued either from *Ripk1*^{fl/fl} or *Ripk1*[LPC-KO] mice, it never induced direct cell death or sensitized hepatocytes to TNF-α. In contrast, it has been demonstrated that in some RIPK1-deficient cell types, such as mouse embryonic fibroblasts or primary human fibroblasts, poly I:C directly induced RIPK3-dependent necroptosis[35−37]. This discrepancy could probably be explained by the limited expression of RIPK3 in hepatocytes[38,39]. Poly I:C-treated animals with RIPK1-deficient hepatocytes developed the usual symptoms of acute liver failure accompanied by an enhanced systemic release of TNF-α, which was responsible for hepatocyte death. Indeed, neutralization of TNF-α by a decoy receptor (ETA) was sufficient to prevent any clinical manifestations in vivo and to protect primary RIPK1-deficient hepatocytes from apoptosis induced by autocrine TNF-α. This pro-inflammatory cytokine turned into a direct death-inducer for hepatocytes when deficient in RIPK1. Thus, the presence of this kinase in hepatocytes protects them from death when TNF-α appears in their microenvironment, whatever the origin of the inflammatory context. Here TNF-α, which emerged from a response to a viral pathogen associated molecular pattern (PAMP)

recognized by the TLR3, was most probably produced by Kupffer cells as their previous chemical depletion prevented the development of hepatitis in poly I:C challenged *Ripk1*[LPC-KO] mice. The protective property of RIPK1 against TNF-α was also observed for immune responses originating from bacterial PAMPs detected by TLR4 and TLR9 (lipopolysaccharides and unmethylated CpG oligodeoxynucleotide, respectively)[24] and in an acute hepatic autoimmune model induced by concanavalin A injection[23]. RIPK1 therefore appears to be an important part of the hepatocyte protection system, especially when the liver undergoes different types of insults, highlighting the potential vulnerability of patients who may harbor already described genetic polymorphism altering the hepatic disease outcome[40] or homozygous amorphic mutations[36].

Materials and methods
Ethics statement
Animal studies were reviewed and approved by the "Comité d'Ethique en Expérimentation Animale" (C2EA – 07) under the French Ministry of Higher Education and Research (permission#: 10460−2017070300162663 v3). The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, EEC Council Directive 2010/63/EU.

Animals, virus, and treatment protocols
For MHV3 experiments, animals were maintained in individually ventilated cages (Forma Scientific, 1 Marietta, OH) in the BSL3 local animal facility. *Ripk1*[LPC-KO] C57BL/6 mice have been already described in previous works[23]. For each experiment, these genetically modified mice were systematically compared to their WT *Ripk1*^{fl/fl} littermates. Homogeneous groups of male and female mice at 8–20 weeks of age were used for each experiment. Genotyping was performed by conventional PCR for Alfp-Cre gene from DNA extracted from tail samples of mice[24].

For in vivo viral inoculation, the pathogenic L2-MHV3 strain were injected by intraperitoneal route at 10^3 50% tissue culture infective dose ($TCID_{50}$) per animal as described previously[41]. Mice were followed up twice a day for weight loss (Supplementary Fig S1A). At 48 h and 72 h post infection, mice were killed by cerebral dislocation before liver and blood sampling. Other mice were followed up for 4 days for survival monitoring.

Poly I:C (Invivogen) diluted in PBS was intravenously administered at a dose of 1.5 mg/kg body weight. Etanercept (ETA, Pfizer) was injected via intraperitoneal route at a dose of 10 mg/kg body weight (10 μL/g body weight) 1 h prior poly I:C challenge. When indicated, macrophages were depleted by liposomes-encapsulated dichloromethylene bis-phosphonate (Cl_2MBP) i.p injections as described previously[24,42]. Briefly, Cl_2MBP was administered twice (with 24 h interval between injections)

in mice. The first and second injections were respectively given at the rate of 10 and then 5 μl/g body weight. Blood was collected before injecting poly I:C, which was injected 48 h after first injection of liposomes-encapsulated Cl$_2$MBP and killed 8 h post poly I:C injection.

Histopathological and biochemical studies

Mouse liver fragments were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry. For histopathology, hematoxylin and eosin (H&E) staining of liver tissues was carried out to investigate the liver injury. Plasma alanine (ALT) and aspartate (AST) transaminases were measured according to the IFCC primary reference procedures using Olympus AU2700 Auto- analyser* (Olympus Optical, Tokyo, Japan).

Immunolocalization in liver tissues

For immunolocalization in liver tissues, paraffin-embedded mouse liver sections (5 μm) were dried for 1 h at 58 °C, followed by antigen retrieval and incubation with primary antibody (anti-cleaved caspase-3, Cell Signaling, #9661; anti-F4/80, eBioscience, #12–4801–80) in a Ventana automated instrument (Ventana Medical Systems, USA). Revelation of primary antibody was carried out using horse-radish peroxidase-conjugated secondary antibody (Dako, USA). All paraffin-embedded mouse liver sections were scanned with a digital slide scanner (Hamamatsu, Nanozoomer 2.0-RS) and files were analysed with the NDP viewer software.

RNA isolation and RT-qPCR

Total RNA was extracted from mice liver tissues and from primary hepatocytes using the NucleoSpin* RNA kit (Macherey-Nagel, #740955). First-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368813, Foster City, CA, USA). Real-time quantitative PCR was performed using the double-strand specific SYBR* Green system (Applied Biosystems, #4367659) on CFX384 Touch™ Real-Time PCR Detection System (Biorad). Each measurement was performed in triplicate. The relative gene expression was normalized against the 18S gene expression. The PBS-treated mice served as reference for mRNA expression (control mRNA level was arbitrarily set at 1). For the absolute quantification of the MHV3 genome by qPCR, a plasmid (pBAC-JHMV^{IA})[43], containing the entire genome of the neurotropic JHM strain of MHV, was used for calibration. The primer sequences are all depicted in Table S1 in supporting information.

Plasma cytokine immunoassay by flow cytometry

Murine cytokines were quantified by bead-based immunoassays according to the manufacturer protocol, using a filter plate and a vacuum filtration system for washing steps (LEGENDplex™ Mouse Inflammation Panel kit, BioLegend). Samples were analyzed on a LSR Fortessa cytometer (BD Biosciences).

Primary hepatocyte isolation and culture

Murine hepatocytes were isolated and purified from adult *Ripk1$^{fl/fl}$* or *Ripk1^{LPC-KO}* mice as described previously[25] with minor modifications. The perfused liver was first washed with solution I (8 g/l NaCl, 0.2 g/l KCl, 0.1 g/l Na$_2$HPO$_4$.12 H$_2$O, 2.38 g/l HEPES, pH 7.6 and 0.5 mM EGTA) at a 10 mL/min flow rate for 8–10 min. Then, the perfusion solution I without EGTA was supplemented with 5 mM CaCl$_2$.2H$_2$O and 0.01% collagenase type 4 (Worthington Biochemical Corporation, Serlabo Technologies, Entraigues, France) at a 7 mL/min flow rate for 5–7 min. After the completed isolation process, hepatocytes were seeded at a density of 6×10^4 cells/cm^2 in 96-well plates and 24-well plates, previously coated with collagen type I (BD Biosciences), in Williams' E medium supplemented with 10% (vol/vol) fetal calf serum, 2 mM glutamine, 10 IU/mL penicillin, 10 μg/mL streptomycin and 5 μg/mL insulin. Around 4 h post plating, cells were washed twice with PBS before their stimulation with poly I:C in a similar medium that the seeding supplemented Williams' E medium in which fetal calf serum was replaced by 1 mg/mL bovine serum albumin with or without 1 μg/mL of ETA (Pfizer). Cell viability was evaluated after a treatment period of 16 h with the Cell Proliferation Reagent WST-1 (Roche), according to the manufacturer's instructions. Cells in 24 wells were processed for RT-qPCR analysis.

Statistical analysis

Data were expressed as means ± SEM for all mice treated similarly. Mean differences between experimental groups were assessed using the non-parametric Mann–Whitney *U*-test. Statistical analysis for the in vitro experiments were performed using the unpaired Student's *t*-test. All statistical analysis was achieved with the GraphPad Prism5 software. Significance is shown as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

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Conflict of interest
The authors declare that they have no conflict of interest.

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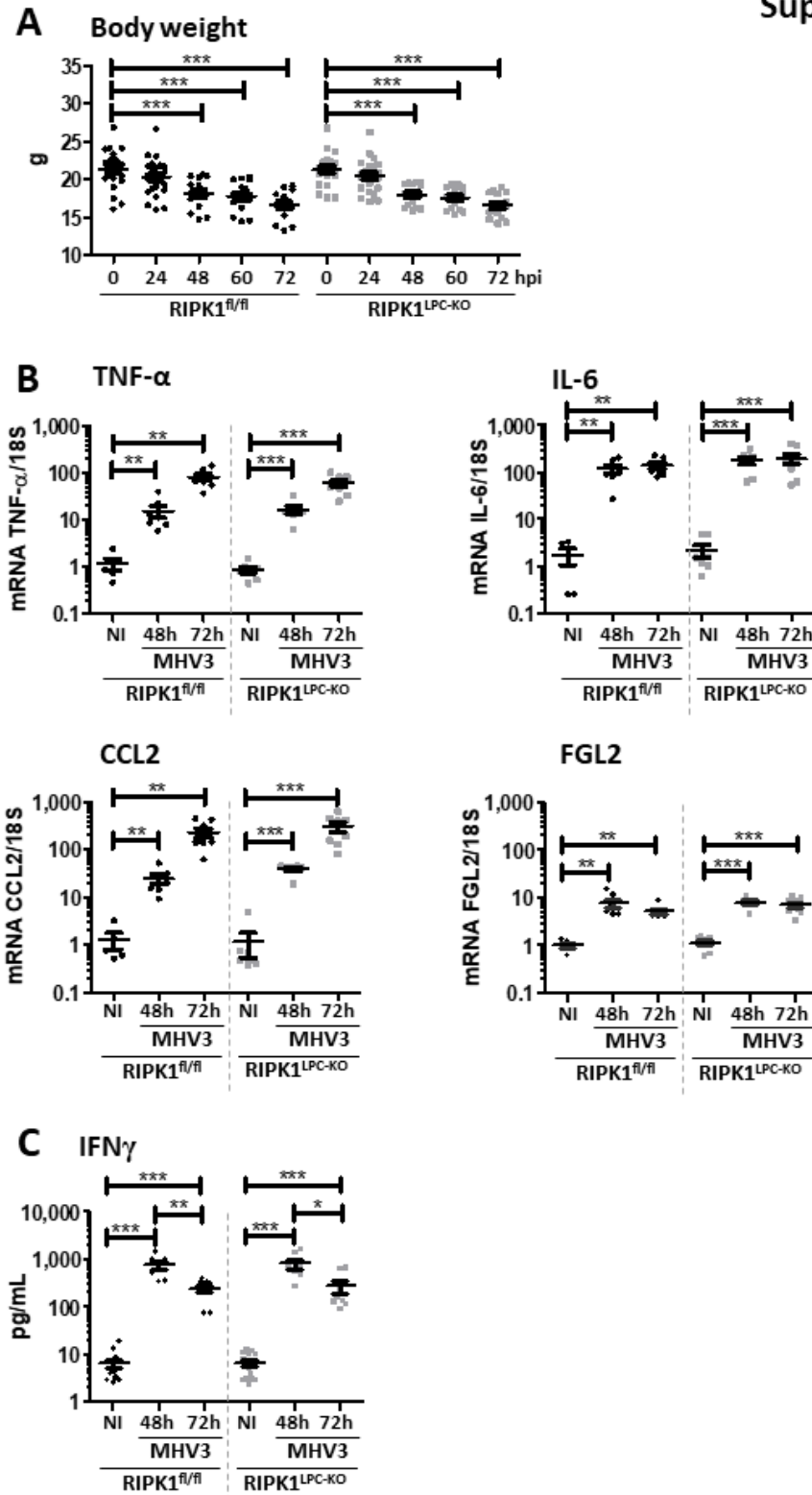
Fig S1. Clinical and molecular parameters in mice inoculated with MHV3. $Ripk1^{fl/fl}$ and $Ripk1^{LPC-KO}$ mice were inoculated or not (NI) with MHV3. Groups of infected animals were euthanized 48 or 72 h post-inoculation. (A) Body weight monitoring after MHV3 inoculation. (B) Levels of hepatic TNF-α, IL-6, CCL2 and FGL2 transcripts. (C) Levels of plasma IFNγ. For all graphs, each black dot and grey square represent a $Ripk1^{fl/fl}$ and a $Ripk1^{LPC-KO}$ individual, respectively, and errors bars are expressed as means ± SEM.

A ALT (72 hpi)   AST (72 hpi)
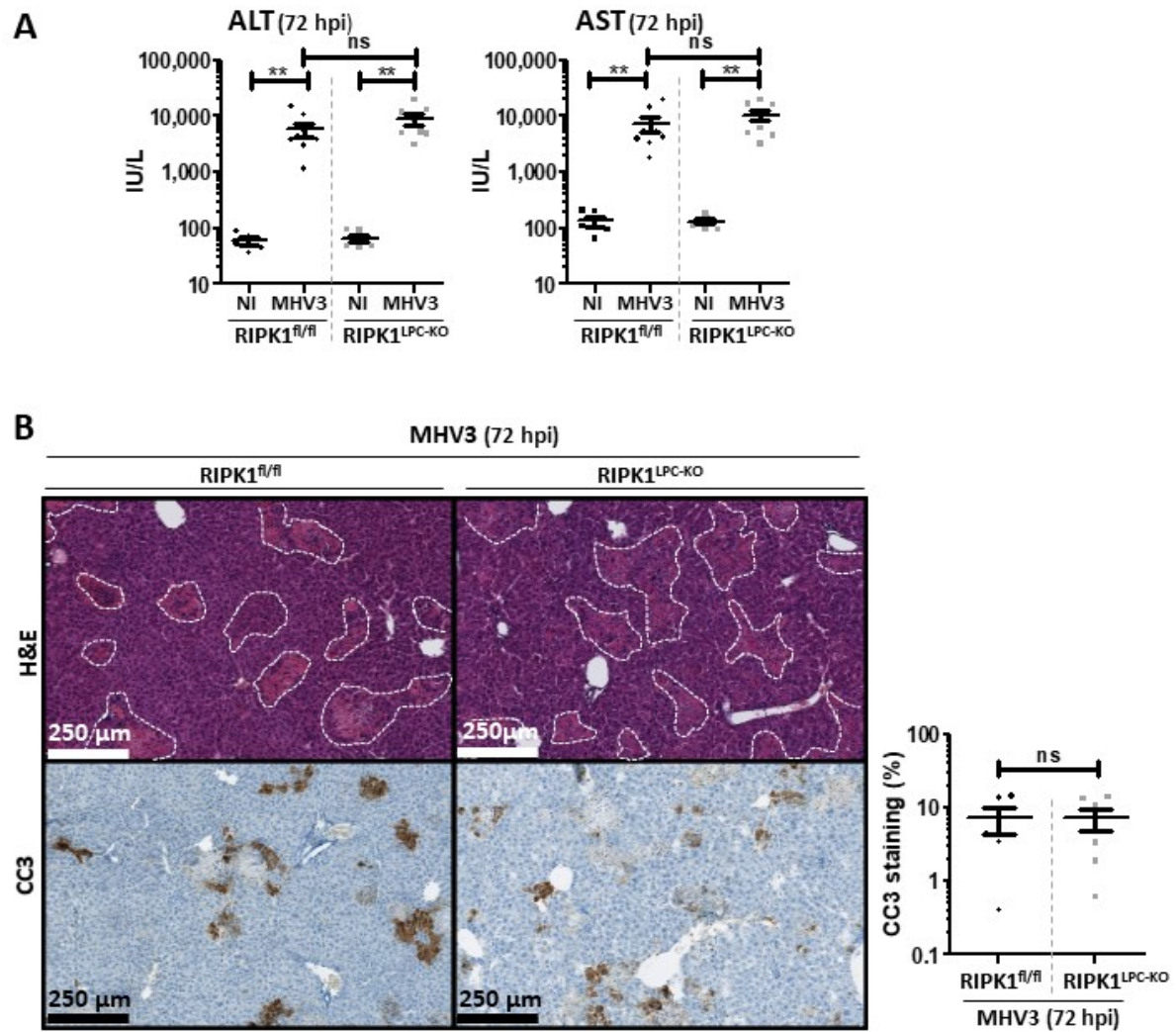
B MHV3 (72 hpi)

Fig S2. Biochemical and histological parameters in mice inoculated with MHV3. *Ripk1*^{fl/fl} and *Ripk1*[LPC-KO] mice were inoculated or not (NI) with MHV3. Groups of infected animals were euthanized 72 h post-inoculation. (A) Levels of plasma ALT and AST. (B) Pictures of liver tissue sections stained by H&E (upper panels, white dashed lines delimit necrotic areas) or analysed by immunohistochemistry for cleaved-caspase-3 (CC3) (lower panels) with signal quantification of cleaved-caspase-3 (low right panel). For all graphs, each black dot and grey square represent a *Ripk1*^{fl/fl} and a *Ripk1*[LPC-KO] individual, respectively, and errors bars are expressed as means ± SEM.
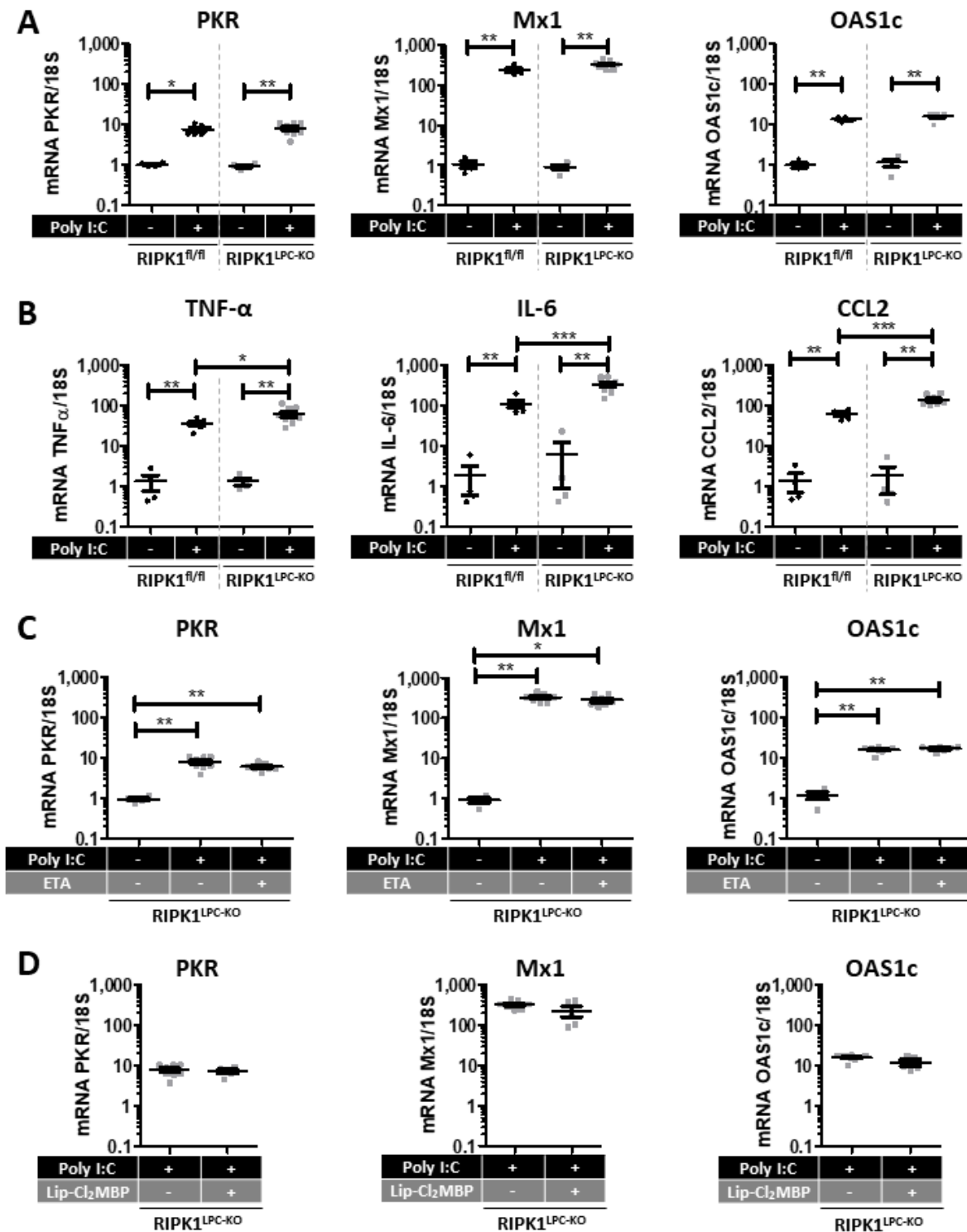
Fig S3. Relative quantification of hepatic transcripts. Levels of hepatic PKR, Mx1, OAS1c (A) and TNF-α, IL-6, CCL2 (B) transcripts in *Ripk1*^{fl/fl} and *Ripk1*[LPC-KO] mice challenged or not by poly I:C. Levels of hepatic PKR, Mx1 and OAS1c transcripts in *Ripk1*^{fl/fl} and *Ripk1*[LPC-KO] mice challenged or not by poly I:C eventually pre-treated by ETA (C) or by liposome-encapsulated Cl2MBP (D). Panels A, C and D are controls for correct poly I:C injections of concerned mice. For all graphs, each black dot and grey square represent a *Ripk1*^{fl/fl} and a *Ripk1*[LPC-KO] individual, respectively, and errors bars are expressed as means ± SEM.

Table S1. Sequences of primers used for qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Tnf	TAGCTCCCAGAAAAGCAAGC	TTTTCTGGAGGGAGATGTGG
Il6	CGATGATGCACTTGCAGA	CTCTGAAGGACTCTGGCTTTG
Tlr3	CACGAACCTGACAGAACTCG	CACTTCGCAACGCAAGG
Eif2ak2 (PKR)	GATGGAAAATCCCGAACAAGGAG	AGGCCCAAAGCAAAGATGTCCAC
Mx1	CCTGGAGGAGCAGAGTGACAC	GGTTAATCGGAGAATTTGGCAA
Oas1c	TAGAATCCATGGCTCCTGCT	AGAATCAGTGCACCACGATG
Rn18s	TTGGCAAATGCTTTCGCTC	CGCCGCTAGAGGTGAAATTC
Alfp-Cre	GCCTGCATTACCGGTCGATGCAACGA	GTGGCAGATGGCGCGGCAACACCATT
MHV3 Capsid	TGGAAGGTCTGCACCTGCTA	TTTGGCCCACGGGATTG

Article 2: RIPK1 depletion in hepatocytes sensitizes to toxic hepatitis induced by CCl4 but not by APAP.

(In Preparation)

Background and aims

APAP-induced liver insult remains the leading cause of drug induced liver injury (DILI) in western world. At high doses, APAP and CCl4 cause acute hepatotoxicity characterized by hepatocyte death. The protein kinase RIPK1 has been described to play kinase-dependent pro-death role but also kinase-independent pro-survival role. Its implication in APAP-poisoning is controversial, but it remains to be discovered with respect to CCl4-induced liver damages. Thus, investigations were designed to study the role of Ripk1 in liver parenchymal cells in APAP- and CCl4- induced hepatotoxicities.

Methods

Mice deficient for RIPK1 specifically in liver parenchymal cells (*Ripk1*^LPC-KO) and their wild-type littermates (*Ripk1*^fl/fl) were exposed to APAP and CCl4. Etanercept, a TNF-α receptor decoy, or Nec-1s (RIPK1 kinase inhibitor) were injected prior to toxic administration to explore the respective role of TNF-α and RIPK1 kinase activity in induced hepatitis. Mice were slaughtered at 24, 48 and 72 hours post-administration of CCL4 and 8 hours post-injection of APAP. Different clinical-pathological investigations (ALT, liver histology, cleaved caspase (CC3) were conducted to analyse challenged animals.

Results

Administration of APAP and CCl4 led to increased serum ALT and AST in both *Ripk1*^fl/fl and *Ripk1*^LPC-KO mice. However, APAP-induced liver damages were similar in both genotypes. Further inhibition of RIPK1 kinase activity in APAP-induced liver injury never improved

marker of liver damage (ALT and AST). Beside, 24 and 48 hours post CCl4 administration, elevations of plasma ALT and AST levels and of cleaved caspase 3 labelling were more pronounced in *Ripk1*^{LPC-KO} mice compared to their wild-type *Ripk1*^{fl/fl} littermates. Otherwise, ALT and AST levels became significantly lower in *Ripk1*^{LPC-KO} mice compared to their wild-type *Ripk1*^{fl/fl} at 72 hours post-administration of CCl4. Pre-injection of ETA had no impact on CCL4-induced hepatitis whatever the genotype and even with increasing concentrations of ETA. Additionally, Nec-1s did not alter the severity of symptoms (plasma ALT and AST levels) in *Ripk1*^{fl/fl} mice.

Conclusion

In conclusion, our results demonstrate that while RIPK1 did not protect, even partially, liver parenchymal cells from APAP toxicity, it ameliorates CCl4 induced hepatotoxicity in TNF-α independent manner.

RIPK1 depletion in hepatocytes sensitizes to toxic hepatitis induced by CCl4 but not by APAP.

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Abstract.

At high doses, APAP and CCl4 induce severe toxic liver insults that can lead to death. Thus, APAP remains the leading cause of drug induced liver injury (DILI) in western world. APAP and CCl4 induce cell death of hepatocytes.

Because RIPK1 is a kinase that can be involved both in cell survival and in death pathways, we aim here to investigate the role of Ripk1 in liver parenchymal cells in APAP induced liver injury and CCl4 induced hepatotoxicity.

Mice deficient for *Ripk1* specifically in liver parenchymal cells (*Ripk1*^{LPC-KO}) and their wild-type littermates (*Ripk1*^{fl/fl}) were exposed to APAP and CCl4. Etanercept, a TNF-α receptor decoy, or Nec-1s (RIPK1 kinase inhibitor) were injected to prior administration of CCl4 and APAP to explore the respective role of TNF-α and RIPK1 kinase activity in induced hepatitis. Different clinico-pathological investigations (liver transaminase measurement, liver histology, Cleaved-Caspase 3 staining) were conducted to analyze challenged animals. Administration of APAP and CCl4 led to increased serum ALT and AST in both *Ripk1*^{fl/fl} and *Ripk1*^{LPC-KO} mice. However, this liver damage remains similar in both *Ripk1*^{fl/fl} and *Ripk1*^{LPC-KO} mice in APAP induced liver damage. Further inhibition of RIPK1 kinase activity in APAP induced liver damage did not improve marker of liver damage (ALT and AST). On the other hand, CCl4 administration resulted in elevated plasma ALT and AST through elevated cleaved caspase 3 labelling in *Ripk1*^{LPC-KO} mice as compared to their wild type *Ripk1*^{fl/fl} littermates. Pre-administration of ETA did not change plasma ALT and AST. Additionally, Nec-1s pretreatment did not alter plasma ALT and AST in *Ripk1*^{fl/fl} mice.

In conclusion, our results demonstrate that RIPK1 did not protect from APAP induced liver damage but ameliorates CCl4 induced hepatotoxicity in TNF-α independent manner.

Introduction

Acetaminophen (paracetamol or N-acetyl-p-aminophenol; APAP) is a widely used anti pyretic and analgesic drug which is listed under the category of over the counter medicines. In adults, FDA recommended therapeutic dose is up to 4 grams daily. Generally, at therapeutic doses, APAP is not toxic, it is metabolized into nontoxic glucuronide and sulfate conjugates. While at higher doses, it can lead to fulminant hepatic failure[1] and APAP induced hepatoxicity is the leading cause of acute liver failure in developed world. APAP is metabolized in hepatocytes by cytochrome P450 to an active toxic metabolite N-acetyl-p-benzoquinone (NAPQI). This NAPQI is detoxified by glutathione (GSH), and APAP-GSH binds to cysteine sulfhydryl group of proteins leading to oxidative stress resulting in rupture of mitochondrial membranes, and death/apoptosis of hepatocytes. APAP induced hepatoxicity depends on various factors like age, sex, nutritional status, genetic background and functioning of liver[1].

Carbon tetrachloride (CCl4), another hepatotoxic agent, is an organic compound which was widely used as a precursor to refrigerants, cleaning agent, degreaser and as a constituent in fire extinguishers until its hepatotoxic and carcinogenic potential was elucidated. Once metabolized in liver, cellular cytochromes convert it into trichloromethyl radical (CCl_3) which can bind cellular proteins, lipids and nucleic acids and interferes in lipid metabolism resulting in steatosis. In the presence of oxygen CCl_3 is further oxidized into CCl_3OO which causes cellular oxidative stress ultimately increasing cell permeability and release of cellular calcium leading to cell death[2].

Both APAP and CCl4 at high doses induce hepatocyte death. Cell death is dependent of a cascade of kinase activation. Thus, c-Jun N-terminal kinases (JNK) is activated through various kinases like receptor interacting protein kinase 1 (RIPK1), mixed lineage protein kinase 3 (MLK3), protein kinase C (PKC), glycogen synthase kinase 3 beta (GSK3 β), apoptosis signal-

regulating kinase 1 (ASK1) and mitogen-activated-protein-kinase kinase 4 (MKK4). The activated JNK activates Sh3 homology3 binding protein5 (Sab), ultimately resulting in amplification of ROS productions causing mitochondrial permeability transition[3,4]. The released contents like endonuclease G and apoptosis inducing factor results in DNA fragmentation. The mitochondrial membrane dysfunction and DNA fragmentation is the main cause of necrotic cell death [5,6]. Absence of cleaved caspase 3 in liver lysates gives the notion of absence of apoptosis [7].

TNF-α is a pro-inflammatory cytokine which play key role in liver diseases. It can bind to its cognate receptors, TNFR1 and TNFR2, although its major functions are carried out through TNFR1 binding. However, its role is controversial in APAP and CCl4 induced hepatotoxicity [2,8-15]. Downstream TNFR1, RIPK1 plays key role in regulation of cell survival and cell death. Previously, we have demonstrated the protective role of RIPK1 in TNF-α induced hepatitis[16]. RIPK1 kinase activity triggers caspase dependent or independent cell death while RIPK1 also plays kinase independent pro-survival role[17]. Inhibition of RIPK1 kinase activity through Necrotstatin-1 (Nec-1) or Nec-1s resulted in amelioration of APAP induced liver injury[18-23]. Furthermore, RIPK1 knockdown through anti-sense oligonucleotide treatment also resulted in amelioration liver injury[18]. In contrast, RIPK1 depletion specifically in liver parenchymal cells does not improve APAP induced liver damage[24]. This discrepancy could be due to different methods employed for RIPK1 depletion or liver non-parenchymal specific role of RIPK1. Acetaminophen and CCl4 pathophysiology in mice are excellent tools to ameliorate the understanding of pathophysiology of human APAP and CCl4 hepatotoxicity respectively. Using mice specifically deficient in RIPK1 in liver non-parenchymal cells, we aimed to explore here the role of kinase activity of RIPK1 in liver non-parenchymal cells in APAP induced hepatoxicity. Furthermore, we also aimed to explore the role of RIPK1 and its kinase activity in CCl4 induced hepatotoxicity as its involvement was suggested in previous studies[25,26].

Results

RIPK1 deficiency in liver parenchymal cells does not change the susceptibility of mice to APAP induced hepatotoxicity

We took advantage of previously demonstrated mice specifically deficient for RIPK1 in liver parenchymal cells. Furthermore, we have also demonstrated that RIPK1 in liver parenchymal cells are dispensable in liver homeostasis[16]. Mice deficient for RIPK1 specifically in liver parenchymal cells (*Ripk1*[LPC-KO]) and their wildtype control (*Ripk1*^{fl/fl} littermates) were administered with APAP at the dose rate of 600mg/kg intraperitoneally. The administration of APAP resulted in significant increase in liver transaminases AST and ALT in *Ripk1*^{fl/fl} mice as well as in *Ripk1*[LPC-KO] as compared to non-injected control mice at 8 hours post injection (hpi) (Figure 1A). However, this increase in liver transaminases was not different in both *Ripk1*[LPC-KO] and *Ripk1*^{fl/fl} mice suggesting RIPK1 is dispersible for APAP induced liver injury. H&E stain shows damaged hepatocytes in both *Ripk1*[LPC-KO] and *Ripk1*^{fl/fl} mice (Figure 1B upper panel). Furthermore, to assess the contribution of apoptosis, we have checked the activation of cleaved caspase-3 (Figure 1B lower panel) which was found to be not activated in APAP injected mice in both *Ripk1*[LPC-KO] and *Ripk1*^{fl/fl} mice.

Nec-1s pre-treatment does not improve liver injury in Ripk*1*[LPC-KO] mice.

Ripk1 depletion by ASO resulted in improvement in liver injury induced by APAP[18]. Furthermore, RIPK1 kinase inhibition by Nec-1 also improved APAP induced liver injury[18,19]. While Ripk1 depletion specifically in liver parenchymal cells does not improve APAP induced hepatotoxicity. Therefore, to check the role of kinase activity of liver non-parenchymal cells, we pre-treated *Ripk1*[LPC-KO] mice with Nec-1s (specific inhibitor of RIPK1 kinase activity) prior to treatment with APAP and were systematically compared to DMSO pre-treated *Ripk1*[LPC-KO] mice. DMSO pre-treatment alone resulted in significant protection in APAP induced liver

injury as suggested by significant decrease in liver transaminases. However, pre-treatment of Nec-1s did not improve APAP induced liver injury in $Ripk1^{LPC-KO}$ mice as evidenced by similar levels of ALT and AST (Figure 2A) in Nec-1s and vehicle (DMSO) pre-treated mice suggesting that RIPK1 kinase activity in liver non-parenchymal cells does not contribute in APAP induced hepatotoxicity, as confirmed by observation of H&E staining (Figure 2B).

RIPK1 deficiency in LPC potentiated CCl4 induced hepatotoxicity

To investigate the role of RIPK1 in CCl4 induced hepatoxicity, both $Ripk1^{LPC-KO}$ and $Ripk1^{fl/fl}$ mice were treated with CCl4 (dissolved in olive oil) orally and liver damage was assessed by transaminases and histopathological staining at 24, 48 and 72 hours post treatment. Irrespective of the genotype, CCl4 treatment resulted in significant increase in release of transaminases (AST and ALT) at 24 hours and continue to rise at 48 hours and decreased drastically at 72 hours. However, release of transaminases was significantly higher at 24 hours in $Ripk1^{LPC-KO}$ mice as compared to $Ripk1^{fl/fl}$ littermates (Figures 3A). Furthermore, H&E staining showed centrilobular liver damage (Figure 3B). Similarly, it remained slightly higher at 48 hours in Ripk1^{LPC-KO} mice as compared to $Ripk1^{fl/fl}$ littermates. Interestingly, the levels of transaminases were significantly lower in Ripk1^{LPC-KO} mice as compared to $Ripk1^{fl/fl}$ littermates at 72 hours post administration of CCl4. CCl4 treatment resulted in centrilobular steatosis in both in $Ripk1^{LPC-KO}$ mice and $Ripk1^{fl/fl}$ littermates. This steatosis and necrotic area tends to increase still 48 hours and decreased thereafter. To investigate, the involvement of apoptosis in CCl4 induced hepatotoxicity, we determined the activation of cleaved caspase 3. Level of cleaved caspase 3 stained area was significantly higher in $Ripk1^{LPC-KO}$ mice as compared to $Ripk1^{fl/fl}$ littermates at 24 hours post treatment (Figure 4. lower panel).

Neutralization of TNF alpha does not ameliorate CCl4 induced hepatotoxicity

Role of TNF-α in CCl4 induced hepatotoxicity remains controversial. To investigate the role of TNF-α, mice were administered ETA (TNF alpha decoy receptor) IP 1 hour prior and 12 hours post administration of CCl4 gavage. To assess liver damage, mice were slaughtered 24 hours post administration of CCl4. Irrespective of the genotype, administration of ETA does not improve liver damage as evidenced by similar level of transaminases with or without ETA (Figure 5).

Pre-treatment of ETA at varying doses does not ameliorate CCl4 induced hepatotoxicity

Previously, it was suggested that protection or potentiation of CCl4 induced hepatotoxicity depends on the dose of ETA used[9]. To test this hypothesis, WT mice were injected with various concentrations of ETA (0.5 mg/kg, 1 mg/kg, 5 mg/kg) prior to treatment of CCl4. Furthermore, ETA treated mice at the dose of 1mg/kg were further divided into two groups, where in 2nd group ETA was repeated after 12 hours with similar dose. The mice were slaughtered 24 hours post CCl4 gavage. Whatever, the dose of ETA used, the liver damage remains similar as evidenced by similar level of transaminases (Figure 6A). Liver damage was further evidenced by H&E staining (Figure 6B).

Neutralization of kinase activity by Nec-1s pre-treatment does not improve CCl4 induced hepatotoxicity

To further assess the role of kinase activity of Ripk1 in CCl4 induced hepatotoxicity, *Ripk1*^{fl/fl} mice were injected with Nec-1s 1 hour prior to the administration of CCl4 and were compared to vehicle (DMSO) treated mice. Liver damage was assessed by release of transaminases. Nec-1s pre-treatment does not improve liver damage as evidenced by similar level of transaminases with or without Nec-1s pre-treatment (Figure 7A). Liver damage is also observed by H&E staining (Figure 7 B).

Discussion

APAP induced hepatotoxicity remains leading cause of drug induced liver injury (DILI) worldwide [1]. Usually, at therapeutic doses, it is not toxic; while at higher dosages, it causes extensive liver damage [27]. Beforehand it was stated that APAP induced liver injury upregulates RIPK1 expression [23]. Downstream of different death ligand receptors, RIPK1 triggers kinase dependent cell death or has kinase independent pro-survival function [28]. RIPK1 through its kinase activity regulates caspase dependent apoptosis or necroptosis by coordinating with RIPK3 and MLKL[29]. Furthermore, it was demonstrated that this liver injury is partially resolved by inhibition of Ripk1 kinase activity through pre-treatment of Necrostatin-1 (Nec-1) [18-23,30]. Usually, necrostatins are dissolved in DMSO. DMSO can interfere in APAP metabolism hence improving liver injury alone and Nec-1 has off targets [31-34]. Therefore, studies using Nec-1 to inhibit RIPK1 kinase activity and absence of DMSO control should be used with caution to investigate role of RIPK1 kinase activity in APAP induced liver damage. Nec-1s is more specific inhibitor of RIPK1 kinase activity and is demonstrated to be efficient in minimizing APAP induced hepatocytes damage in vitro and in-vivo[23]. In accordance with previous data of Schneider et al [24], we have demonstrated that Ripk1 depletion specifically in liver parenchymal cells does not contribute in APAP induced hepatotoxicity [24]. Additionally, knockdown of Ripk1 through antisense oligonucleotide treatment efficiently reduced liver damage in vivo demonstrating the protective role of Ripk1 protein in APAP induced liver damage[18]. These conflicting results could be due to involvement of Ripk1 in liver non-parenchymal cells or difference in method employed to deplete *Ripk1*. In current study we have demonstrated that additional inhibition of Ripk1 kinase activity in *Ripk1*[LPC-KO] mice does not contribute to APAP induced liver injury. Difference in susceptibility of genetically and pharmacologically depletion of *Ripk1* to APAP induced hepatotoxicity can be due to different methods employed to deplete

Ripk1 as genetically depleted mice were never had Ripk1 expression while ASO depleted Ripk1 transiently. Furthermore, in agreement with previous studies we have revealed that the mode of cell death in APAP induced hepatotoxicity is independent of caspase activation [35] and Ripk1 deficiency in liver parenchymal cells does not switch to apoptotic cell death as in ConA induced liver damage [16]. In addition, mice deficient for RIPK3 or MLKL are not protected from APAP induced liver damage suggesting the absence of necroptosis[18]. Collectively this gives the notion that Ripk1 regulates necrotic cell death independent of apoptosis and necroptosis, which is most probably due to kinase activity of RIPK1 in liver parenchymal cells.

Downstream of death ligands and PAMPs signalling, RIPK1 also interacts with RIPK3 to form necrosome ultimately leading to necroptosis. RIPK1 interaction with RIPK3 increases with repeated injection of CCl4 suggesting the possible involvement of RIPK1 in CCl4 induced hepatotoxicity [26]. Here we demonstrated that RIPK1 depletion specifically in liver parenchymal cells sensitizes $Ripk1^{LPC-KO}$ mice to CCl4 induced hepatocyte apoptosis at 24 hours post administration. Interestingly the level of transaminases remains significantly elevated in $Ripk1^{fl/fl}$ as compared to $Ripk1^{LPC-KO}$ mice at 72 hours. This could possibly be explained by the hypothesis that RIPK1 deficient hepatocytes died earlier in response to CCl4 while in RIPK1 competent hepatocytes damage is bit delayed.

Increased hepatic expression of liver transcript of death ligand (TNF-α) and elevated plasma concentrations of TNF-α are consistent features of CCl4 induced hepatotoxicity [36-38] and RIPK1 plays protective role in TNF-α induced hepatitis [16,39]. Although TNF-α is involved in various liver pathologies [40] the role of TNF-α remains controversial in CCl4 induced hepatotoxicity [2]. Mice deficient for TNFR1 or TNFR2 have similar liver damage in acute CCl4 induced hepatotoxicity [41,42] suggesting that TNF signalling pathway is not decisive in CCl4–induced hepatitis. In contrast other studies demonstrated that depletion of TNFR1 or neutralization of

TNF-α could play protective role [8]. However, in our settings the increased sensitivity of *Ripk1*^{LPC-KO} to CCl4 induced hepatotoxicity is TNF-α independent as pre-treatment with ETA does not improve liver injury. Previous reports suggest that varying dose of ETA can have protective or damaging effect [9]. In contrast, we demonstrate that even varying doses of ETA do not change the susceptibility of mice to CCl4 induced liver toxicity. These conflicting reports on the role of TNF-α or its cognate receptors could be due to dose and route of administration of CCl4, time of slaughtering post administration of CCl4, different strain or species of rodents. Additionally, commensal microbiota could affect the susceptibility of mice to CCl4 induced liver damage in chronic settings[43] and have also been describe to be involved in acute liver injury [44].

FASL is also involved in CCl4 induced hepatotoxicity as mice deficient for FASL or neutralization of FASL resulted in improved liver injury [37]. On the other hand, RIPK1 plays protective role in JO$_2$ (anti-FAS antibody) induced liver damage [45]. This could explain the increased sensitivity of *Ripk1*^{LPC-KO} to CCl4 induced hepatotoxicity.

Materials and Methods

Animals, treatment protocols

Ripk1^LPC-KO C57BL/6 mice have been already described in previous works. For each experiment, these genetically modified mice were systematically compared to their WT *Ripk1* littermates. Homogeneous groups of adult female mice were used for each experiment. Genotyping was performed by conventional PCR for Alfp-Cre gene from DNA extracted from tail samples of mice. CCl4 diluted in olive oil was administered orally at a dose of 2.4 g/kg body weight (10 µL/g body weight). Etanercept (ETA, Pfizer) was injected via intraperitoneal route at a dose of 10 mg/kg body weight (10 µL/g body weight) 1 h prior to CCl4 challenge. Nec-1s Biovision Inc., Milpitas, CA, USA, #2263) dissolved in 5,6% DMSO final was administered by i.v. injection (6.25 mg/kg and 4 µl/g body weight) 1 hour before the CCl4 challenge. For nec-1s experiments, DMSO 5.6% pre-treated mice were used as control.

Acetaminophen (APAP) diluted in pre-warmed PBS, was administered intraperitoneally at a dose of 600 mg/kg body weight. Nec-1s Biovision Inc., Milpitas, CA, USA, #2263) dissolved in 5,6% DMSO final, was administered by i.v. injection (6.25 mg/kg and 4 µl/g body weight) 1 hour before the APAP challenge. For nec-1s experiments, DMSO 5.6% pre-treated mice were used as control.The mice were slaughtered at indicated time points. Olive oil or PBS administered age and sex matched mice were used as control for CCl4 and APAP experiments respectively.

Histopathological and biochemical studies

Mouse liver fragments were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry. For histopathology, hematoxylin and eosin (H&E) staining of liver tissues was carried out to investigate the liver injury. Plasma alanine (ALT) and aspartate (AST)

transaminases were measured according to the IFCC primary reference procedures using Olympus AU2700 Auto- analyser[®] (Olympus Optical, Tokyo, Japan).

Immunolocalization in liver tissues

For immunolocalization in liver tissues, paraffin-embedded mouse liver sections (5 µm) were dried for 1 h at 58°C, followed by antigen retrieval and incubation with primary antibody (anti-cleaved caspase-3, Cell Signaling, #9661 in a Ventana automated instrument (Ventana Medical Systems, USA). Revelation of primary antibody was carried out using horse-radish peroxidase-conjugated secondary antibody (Dako, USA). All paraffin-embedded mouse liver sections were scanned with a digital slide scanner (Hamamatsu, Nanozoomer 2.0-RS) and files were analysed with the NDP viewer software.

Statistical analysis

Data was expressed as means ± SEM for all mice treated similarly. Mean differences between experimental groups were assessed using the non-parametric Mann–Whitney U-test. All statistical analysis was achieved with the GraphPad Prism5 software. Significance is shown as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

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Conflict of interest: The authors declare no conflict of interest.

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LEGENDS

Figure 1. Deficiency of RIPK1 in liver parenchymal cells does not sensitize mice to APAP induced hepatotoxicity. A) Levels of plasma ALT and AST in $Ripk1^{fl/fl}$ and $Ripk1^{LPC-KO}$ mice treated with control (PBS) or APAP at 600mg/kg at 8 hours post injection. **B)** H&E stained liver sections (upper panel) and cleaved caspase 3(CC3) IHC (lower panel). For all graphs, each dot represents an individual and errors bars are expressed as means ±SEM. (*P<0.05; **P<0.01; ***P<0.001; ns, non-significant).

Figure 2. Inhibition of RIPK1 kinase activity by pre-treatment of Nec-1s in $Ripk1^{LPC-KO}$ mice does not protect from APAP induced liver damage. A) Levels of plasma ALT and AST in $Ripk1^{LPC-KO}$ mice treated with APAP at 600mg/kg at 8 hours post injection pre-treated with DMSO or Nec-1s. **B)** H&E stained liver sections. For all graphs, each dot represents an individual and errors bars are expressed as means ±SEM. (*P<0.05; **P<0.01; ***P<0.001; ns, non-significant).

Figure 3. Deficiency of RIPK1 in liver parenchymal cells sensitizes mice to CCl4 induced hepatotoxicity. A) Levels of plasma ALT and AST in $Ripk1^{fl/fl}$ and $Ripk1^{LPC-KO}$ mice treated with control (olive oil) or CCl4 at 2.4g/kg at 24, 48 and 72 hours post gavage. **B)** H&E stained liver sections. For all graphs, errors bars are expressed as means ±SEM. ($,*P<0.05; $$,**P<0.01; $$$,***P<0.001; ns, non-significant).* is used for comparisons within same genotype with reference to non-treated control and $ is used for the comparisons within same treatment group between genotypes.

Figure 4. Deficiency of RIPK1 in liver parenchymal cells sensitizes mice to CCl4 induced apoptosis. CC3 stained liver sections and quantification of CC3 stained area (lower panel).

For all graphs, each dot represents an individual and errors bars are expressed as means ±SEM. (*P<0.05; **P<0.01; ***P<0.001; ns, non-significant).

Figure 5. Pre-treatment of ETA (TNF-α decoy receptor) does not protect from CCl4 induced liver damage. Levels of plasma ALT and AST in ETA pre-treated (at dose rate of 10mg/kg in two doses; 1st one hours before CCl4 gavage and 2nd 12 hours post CCl4 gavage) in *Ripk1*fl/fl and *Ripk1*LPC-KO mice treated with CCl4 at 2.4g/kg at 24 hours post gavage. For all graphs, each dot represents an individual and errors bars are expressed as means ±SEM. (*P<0.05; **P<0.01; ***P<0.001; ns, non-significant).

Figure 6. Pre-treatment of ETA (TNF-α decoy receptor) at varying doses does not protect from CCl4 induced liver damage. A) Levels of plasma ALT and AST in ETA pre-treated in *C57BL/6J* mice treated with CCl4 at 2.4g/kg at 24 hours post gavage. * indicates same dose of ETA repeated after 12 hours. **B)** H&E staining of liver section. For all graphs, errors bars are expressed as means ±SEM. (*P<0.05; **P<0.01; ***P<0.001; ns, non-significant).

Figure 7. Inhibition of kinase activity of RIPK1 by pre-treatment of Nec-1s does not protect from CCl4 induced liver damage. A) Levels of plasma ALT and AST in *Ripk1*fl/fl mice treated with CCl4 at 2.4g/kg at 24 hours post injection pre-treated with DMSO or Nec1s.**B)** H&E staining of liver section. For all graphs, each dot represents an individual and errors bars are expressed as means ±SEM. (*P<0.05; **P<0.01; ***P<0.001; ns, non-significant.

Figure 1

A

B
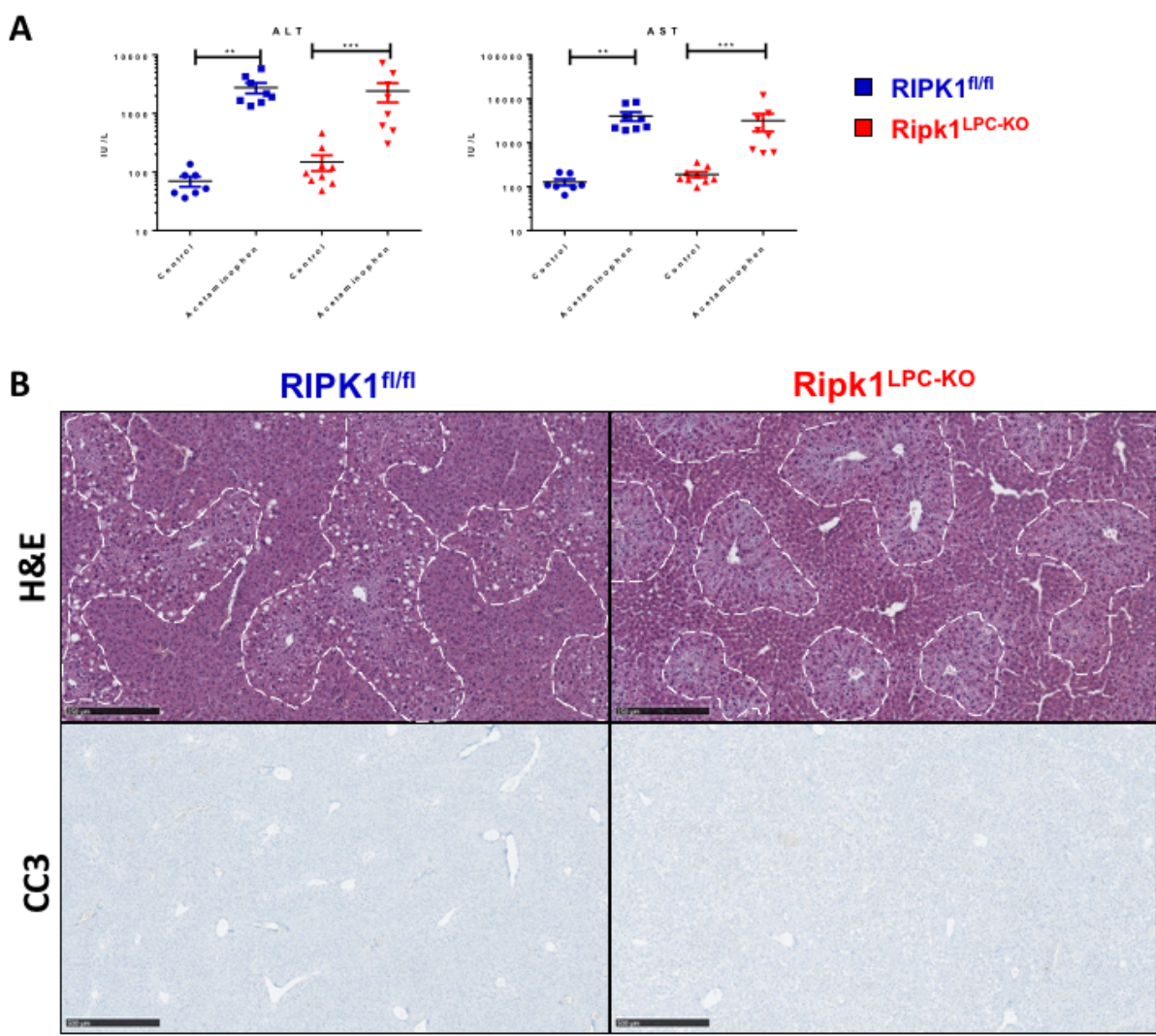
Acetaminophen 600mg/kg body weight
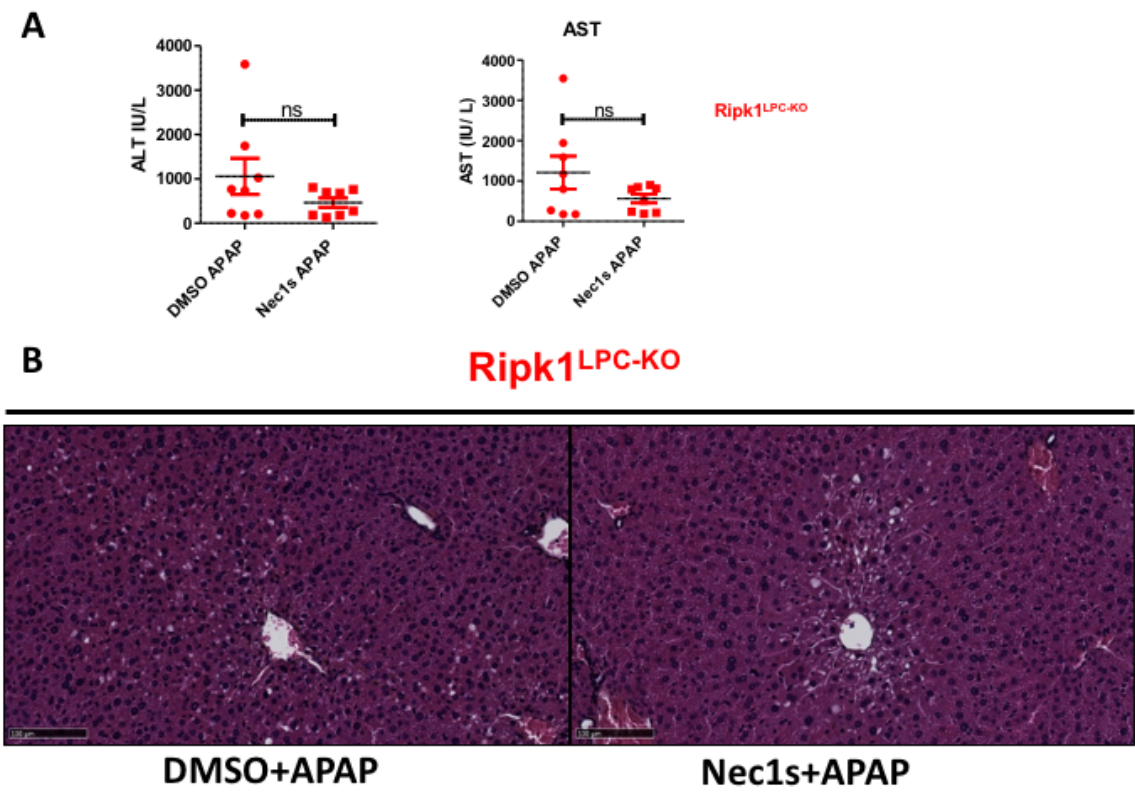
Figure 2

A
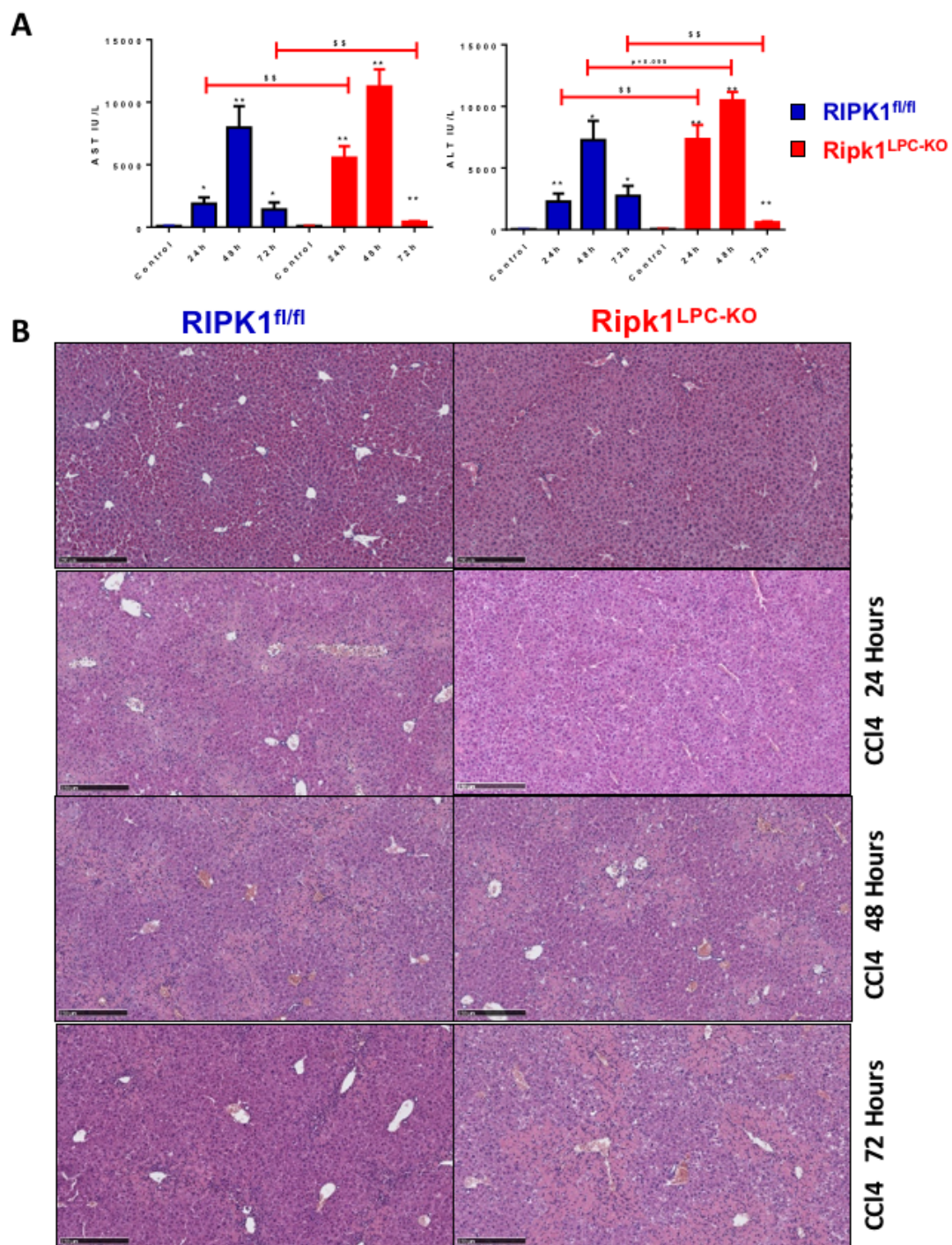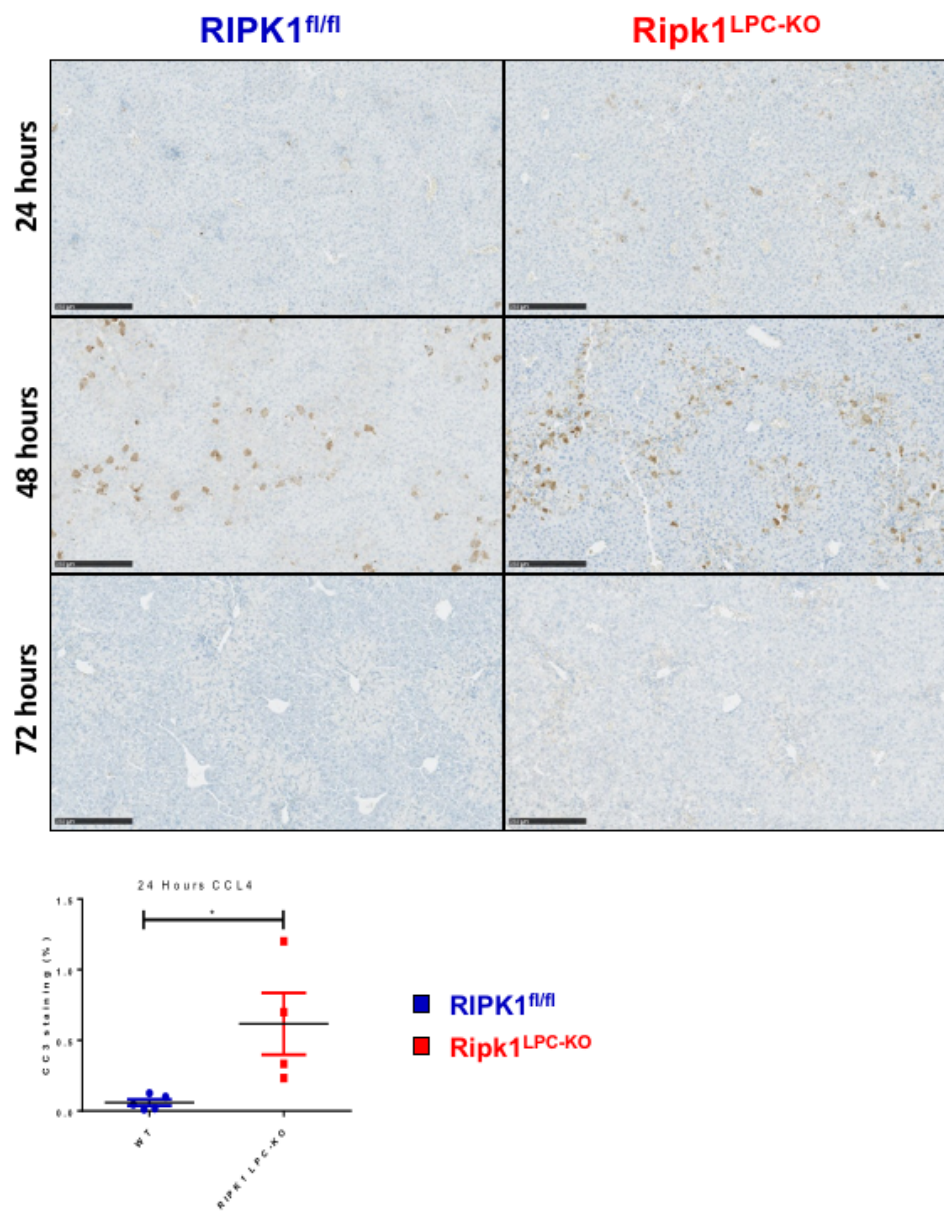

B

Ripk1LPC-KO

DMSO+APAP   Nec1s+APAP

Figure 3
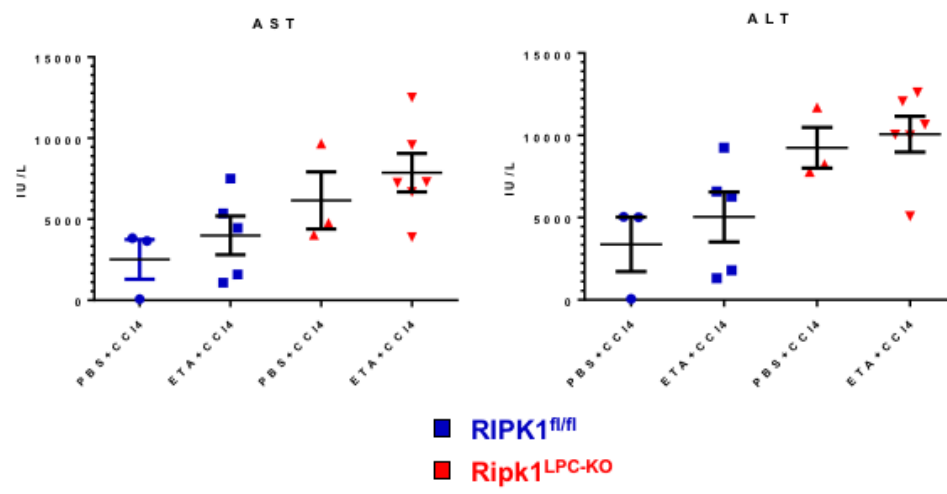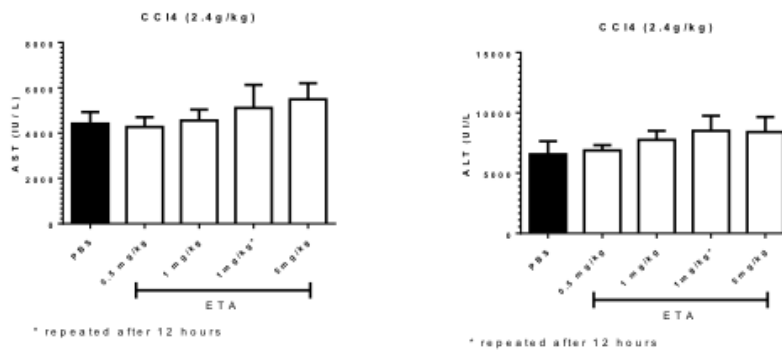
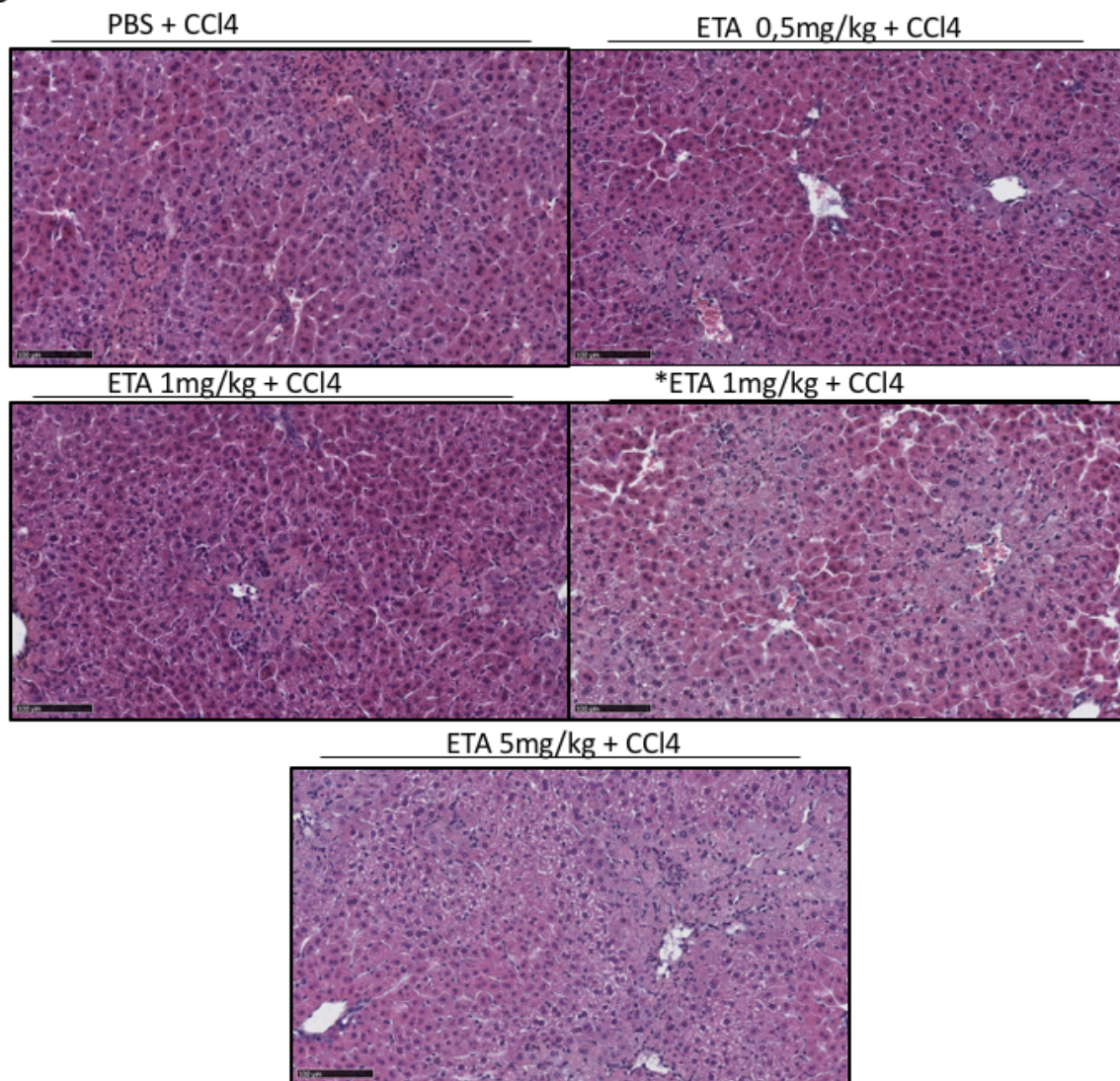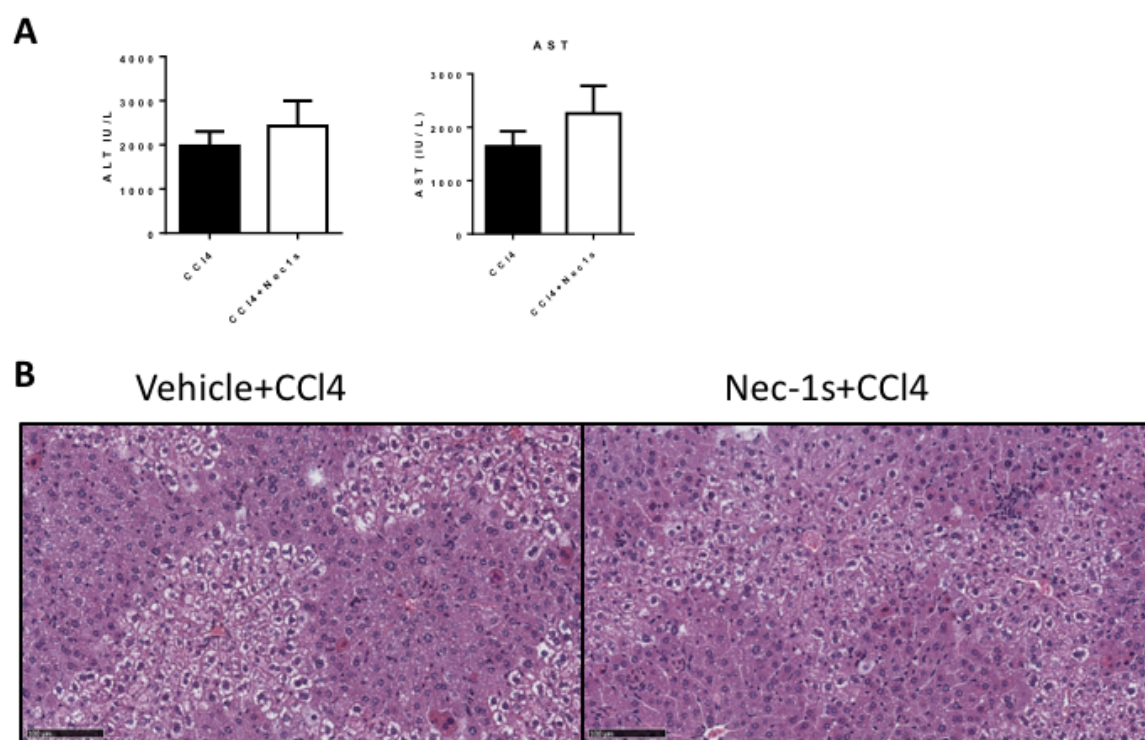Figure 4

Figure 5

Figure 6

A

Figure 7

A

B

Article 3. Ablation of RIPK1 in liver parenchymal cells exacerbates liver fibrosis in High Fat Diet induced NASH in mice.

(In preperation)

Background and aims

Non-alcoholic steatohepatitis (NASH) is an emergent chronic liver disease with high prevalence in occidental countries. NASH can further progress into liver fibrosis, cirrhosis and hepatocellular carcinoma. Hepatocyte death induced by different death ligands plays key role in fibrotic progression. Previously, we showed that RIPK1, a protein kinase known to be involved in pathways related to both cell survival and death, exhibits a protective role in TNFα- and FASL-induced hepatocyte death. Our new study aimed to investigate the role of RIPK1 in NASH.

Method

To decipher the role of RIPK1 in NASH, we took advantage of $Ripk1^{LPC-KO}$ mice which are deficient for RIPK1 only in liver parenchymal cells. NASH was induced by feeding both $Ripk1^{LPC-KO}$ animals and their WT (Ripk1$^{fl/fl}$) littermates with High Fat Diet (HFHCD). Mice were slaughtered at 3, 5 and 12 weeks of HFHCD feeding and were compared to mice fed with normal chow diet. Cytometry was performed to analyze the recruitment of inflammatory cells during the course of NASH. Plasma cytokine levels were measured by LEGENDplex™. Expression modulation of inflammatory genes and genes involved in fibrosis was investigated by RT-QPCR. Liver damage was assessed by histological staining and serum transaminase dosages. Liver fibrosis was quantified after Sirius red labelling.

Results:

 HFHCD feeding for 12 weeks resulted in increased expressions of liver transcripts of RIPK1. Furthermore, this was accompanied by increased expression of death ligands (TNF, FASL,

TRAIL) and of their associated receptors (TNFR, FAS, TRAIL-R). Additionally, irrespective of their genotype, HFHCDD feeding induced progressive hepatomegaly and liver damage, as evidenced by ratio of liver weight to body weight and by plasma level of ALT, respectively. On histological examination, HFHCD-fed mice showed inflammatory infiltrates and hepatocyte ballooning. Huge infiltrations of inflammatory cells (macrophages, NK cells, NKT cells, neutrophils, lymphocytes) were detected at 3 weeks in both *Ripk1*^{LPC-KO} mice and WT littermates. Similarly, irrespective of the genotype, after 5 and 12 weeks of treatment, the number of total immune cells decreased with a total loss of NKT cells. However, some cells such as macrophages and cytotoxic lymphocytes remained over-represented. This inflammation and immune cell infiltration were accompanied by higher plasma doses of cytokines such as TNFα and CCL2. IL-27 and IFNγ were specifically increased at week 3 of HFHCD. Despite similar inflammatory responses, more fibrosis was significantly evidenced by increased sirius red positive area at week 12 in *Ripk1*^{LPC-KO} mice as compared to their WT littermates. This finding was further supported by more elevated mRNA expression of TGFBi and TIMP2 (genes indicative of fibrosis) in *Ripk1*^{LPC-KO} mice.

Conclusion:

Our results show that RIPK1 in liver parenchymal cells has a tendency to limit the progression of liver fibrosis in HFHCD-induced NASH in mice.

Ablation of RIPK1 in liver parenchymal cells exacerbates liver fibrosis in High Fat Diet induced NASH in mice.

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Abstract

Non-alcoholic steatohepatitis (NASH), a chronic liver disease, is 2nd leading cause of liver

transplantation in USA and predicted to be the leading cause in next decade. NASH can further

progress into liver fibrosis, cirrhosis and hepatocellular carcinoma. Hepatocyte death carried through different death ligands plays key role in this progression. Previously, we showed that RIPK1 in hepatocytes exhibits a protective role in this ligand-induced death. Our new study aims to investigate the role of RIPK1 in NASH.

To decipher the role of RIPK1 in NASH, we took advantage of $Ripk1^{LPC-KO}$ mice which are deficient for RIPK1 only in liver parenchymal cells. NASH was induced by feeding both $Ripk1^{LPC-KO}$ animals and their WT ($Ripk1^{fl/fl}$) littermates with High Fat Hight Cholestrol Diet (HFHCD). Mice were slaughtered at 3, 5 and 12 weeks of HFHCD feeding and were compared to mice fed with normal chow diet. Cytometry was performed to analyze the recruitment of inflammatory cells during the course of NASH. Plasma cytokine levels were measured by Legend Plex. Liver damage was assessed by histological staining and serum transaminase dosages. Liver fibrosis was quantified by Sirius red labelling. Liver transcript expression was assessed by qPCR. Irrespective of their genotype, mice fed with HFHCD, showed elevated levels of ALT and liver to body weight ratio during the development of NASH. Important liver infiltrations of immune cells, including NKT cells, were detected at week 3 of HFHCD. Then, after 5 and 12 weeks of treatment, the number of total immune cells decreased with a total loss of NKT cells. However, some cells such as macrophages and cytotoxic lymphocytes remained over-represented. This inflammation and immune cell infiltration were accompanied by higher plasma doses of cytokines such as TNF alpha and CCL2. IL-27 and IFN beta were specifically increased at week 3 of HFHCD. Despite similar inflammatory responses, more fibrosis was significantly evidenced at week 12 in $Ripk1^{LPC-KO}$ as compared to their WT littermates. These findings were further supported by more elevated mRNA expression of TGFBi and TIMP2 (genes indicative of fibrosis) in $Ripk1^{LPC-KO}$ compared to WT mice.

In conclusion, our results show that RIPK1 in hepatocyte limits the progression of liver fibrosis during NASH.

Ablation of RIPK1 in liver parenchymal cells exacerbates liver fibrosis in High Fat Diet induced NASH in mice.

INTRODUCTION

The evolution to a sedentary lifestyle, also accompanied by more caloric diet intakes, is responsible for the emergence of a new silent epidemic in human. Indeed, nearly a quarter of the world's population, with some geographical disparity, would be affected by non-alcoholic fatty liver diseases (NAFLD) (*1, 2*). These cover a spectrum of hepatic disorders ranging from benign steatosis to inflammatory status, defined as non-alcoholic steatohepatitis (NASH), responsible for the development of fibrosis that will eventually worsen, leading to cirrhosis and even hepatocellular carcinoma (HCC) with high rates of morbidity and mortality (*3*). NASH is currently the second leading cause of liver transplantation in USA after hepatitis C and is expected to predominate in the future among the US and European population (*4*).

Although influenced by genetic and epigenetic factors, the pathogenesis of NAFLD would result from multiple concomitant insults originating from gut and adipose tissue. Thus, a "multiple parallel hits" theory has recently been proposed to explain the NAFLD pathogenesis leading to NASH (*5, 6*). At this pathological stage, an inflammatory state is established due to the stress experienced by hepatocytes. In parallel, hepatocyte death is compensated by the activation of a regeneration process. Repetition of these events over time eventually leads to liver tissue remodelling, characterized by the development of fibrosis. Thus, activated Kupffer cells (KCs), infiltrating macrophages and damaged hepatocytes release various cytokines that activate quiescent hepatic stellate cells (HSCs) in collagen-secreting myofibroblasts (*7*). This fibrosis results from an imbalance characterized by an overproduction of extracellular matrix (ECM) (*8*) and an exacerbated inhibition of the HSCs and KCs secreted matrix metalloproteinases (MMPs) by different specific endogenous tissue inhibitors of metalloproteinases (TIMPs) (*9, 10*).

Hepatocyte death fuels the inflammation that causes liver fibrosis and eventually promotes HCC development when chronic (*11, 12*). The death factors TNF-α, Fas Ligand and TNF-related apoptosis-inducing ligand (TRAIL), are found overexpressed in liver tissue during NASH (*13*). Cellular responses induced by these members of the TNF superfamily engage the receptor interacting protein kinase-1 (RIPK1)(*14-16*). Within the intracellular transduction pathway specifically triggered by TNF-α, RIPK1 directs signalling to a pathway of cell survival or death, either by caspase-dependent apoptosis or by RIPK3/MLKL-dependent necroptosis. The role of this molecular switch between cell survival and death has been widely investigated during acute hepatitis. Thus, in viral or autoimmune hepatitis murine models, RIPK1 expressed in hepatocytes promotes their survival in a microenvironment rich in death ligands. (*17-19*). During NASH, the associated dysfunction of the intestinal barrier leads to significant leaks of pro-inflammatory bacterial pathogen associated molecular patterns (PAMPs) to which the liver is exposed (*20-23*). We and others have demonstrated that RIPK1 expressed in hepatocytes protects them from TNF-dependent death in an inflammatory context induced by PAMPs (*24-26*). In this study, we investigated the functions of RIPK1 in mouse hepatocytes in the context of high fat high cholesterol diet induced NASH, mimicking human disease (*27*).

Results

HFHCD induced expression of death ligands, their cognate receptors and Ripk1

Cell death is key event in the progression of NASH. To evaluate the expression of death ligands and their receptors in NASH, WT mice were fed HFHCD for 12 weeks and were systematically compared to age and sex matched standard chow fed mice. HFHCD feeding resulted in increased expression of liver transcripts of death ligands (TNF alpha, FAS L and TRAIL) as well as their cognate receptors (TNFR1, TNFR2, FAS and DR5) (Figure 1A). Furthermore, HFHCD feeding also resulted in increased expression of liver transcript of Ripk1 (Figure 1B).

HFHCD diet causes Hepatomegaly and liver damage irrespective of the genotype.

To investigate the role of RIPK1 in NASH, we took advantage of previously reported mice deficient for RIPK1 specifically in liver parenchymal cells (*Ripk1*LPC-KO) and were compared systematically to their wild type (*Ripk1*fl/fl) littermates. The HFHCD feeding resulted in progressive liver damage in a time dependent manner at 3, 5 and 12 weeks post HFHCD feeding irrespective of the genotype as indicated by elevated liver transaminases (ALT and AST) (Fig 2A upper panel). Furthermore, the HFHCD feeding resulted in progressive hepatomegaly as compared to their standard chow diet control (Fig 2A middle panel). This hepatomegaly did not vary between both *Ripk1*LPC-KO and *Ripk1*fl/fl mice. Hepatocyte ballooning with addition to recruitment of inflammatory cells is observed by H&E as early as 3 weeks post HFHCD feeding (Figure 2B). It is important to note that HFHCD feeding did not induced obesity, this could be due to composition of diet with 85.7% of fat and very low palatability (Fig. 2A, lower panel).

HFHCD feeding causes significant changes in liver inflammation during development of NASH in Ripk1 independent manner

Irrespective of the genotype, steatosis is accompanied by huge recruitment of inflammatory cells in liver at 3 weeks post HFHCD feeding which decreased at 5 and 12 weeks post HFHCD feeding. Therefore, we further performed cytometry to characterize the significant changes in liver immune populations among lymphoid and myeloid cells during the course of development of NASH (Fig 5A). All immune cells from myeloid (neutrophils and macrophages) and lymphoid (LT4, LT8, LB, NK, NKT, Tregs) origin were significantly higher at 3 weeks post HFHCD feeding which started to diminish afterwards. As we have reported previously (*28*), where, the NKT cells were almost depleted at 12 weeks post HFHCD feeding. Next, we aimed to check CD69 expression (marker for activation of immune cells), all the immune cells were

activated at 5 weeks post HFHCD feeding. However, this immune cell activation remains similar in both phenotypes.

Furthermore, we checked the level of different cytokines during the course of development of NASH in both *Ripk1*^{LPC-KO} and *Ripk1*^{fl/fl} mice. Among 13 cytokines assessed, the plasma concentration of IFNβ and IL-27 significantly increased at 3 weeks in both *Ripk1*^{LPC-KO} and *Ripk1*^{fl/fl} mice post HFHCD feeding and returned to normal level thereafter. Furthermore, we investigated the level of pro-inflammatory cytokine TNFα and chemokine CCL2. The plasma concentration of both TNFα and CCL2 started to increase progressively. Irrespective of the genotype, the liver transcript of both TNFα and CCL2 showed significant progressive upregulation in both *Ripk1*^{LPC-KO} and *Ripk1*^{fl/fl} mice.

RIPK1 protects from HFHCD induced fibrosis in NASH

To investigate the role of RIPK1 in liver fibrosis, the Sirius red staining was performed on liver sections from HFHCD and SD fed mice and showed a progressive increase starting from 5 weeks HFHCD in both *Ripk1*^{LPC-KO} and *Ripk1*^{fl/fl} mice (Figure 6B). The Sirius red stained area after 12 weeks post HFHCD feeding was significantly higher in *Ripk1*^{LPC-KO} as compared to their *Ripk1*^{fl/fl} littermates (Figure 6A, lower right panel).

Biomarkers of liver fibrosis as TGFβ induced (TGFBI) was significantly upregulated in HFHCD feeding in a time dependent manner in both *Ripk1*^{LPC-KO} and *Ripk1*^{fl/fl} mice. However, the liver transcript expression of TGFBI was significantly higher in *Ripk1*^{LPC-KO} mice as compared to their wild type *Ripk1*^{fl/fl} littermates.

The study of expression of the expression of genes involved in production and degradation of ECM as α SMA (marker of activation of hepatic satellite cells) and Collagen 1α, started to significantly upregulate at 3 weeks and continue to rise in time dependent manner in both

Ripk1^{LPC-KO} and *Ripk*1^{fl/fl} mice. Nevertheless, RIPK1 does not interfere with expression of liver transcripts of both α SMA and Collagen 1α.

Liver transcript of both Timp 1 and Timp 2 (2 inhibitors of MMPs) were started to increase at 3 weeks post HFHCD feeding in both *Ripk1*^{LPC-KO} and *Ripk1*^{fl/fl} mice and continue to increase thereafter (Figure 6A). Their levels were slightly higher in *Ripk1*^{LPC-KO} mice at 12 weeks post HFHCD feeding as compared to their wild type *Ripk1*^{fl/fl} littermates. The liver transcript level of Timp 2 was significantly higher at 5 weeks post HFHCD feeding in *Ripk1*^{LPC-KO} mice as compared to their wild type *Ripk1*^{fl/fl} littermates. Liver transcripts of MMP-2 remained at basal level at 3 weeks post HFHCD feeding and started to increase at 5 weeks and continue to rise thereafter in both *Ripk1*^{LPC-KO} and *Ripk1*^{fl/fl} mice. Liver transcript of MMP-2 were significantly higher in Ripk1^{LPC-KO} mice as compared to their wild type *Ripk1*^{fl/fl} littermates (Figure 6A). Irrespective of the genotype, liver transcript of Mmp-13 started to increase at 3 weeks post HFHCD feeding and continue to rise until 5 weeks post HFHCD feeding and started to diminish thereafter. Its level were significantly lower in *Ripk1*^{LPC-KO} mice at 12 weeks post HFHCD feeding as compared to 5 weeks post HFHCD feeding. At 12 weeks post HFHCD feeding, its level was significantly lower in *Ripk1*^{LPC-KO} mice as compared to their wild type *Ripk1*^{fl/fl} littermates. Furthermore, markers of oxidative stress as liver transcript of Nox-2 was upregulated progressively in both genotypes. However, at 12 weeks post HFHCD feeding, its expression remained significantly higher in *Ripk1*^{LPC-KO} mice as compared to their wild type *Ripk1*^{fl/fl} littermates (Figure 7A). In conclusion, depletion of *Ripk*1 sensitizes to increased liver fibrosis in HFHCD induced NASH in mice.

Material and Methods

Animals

Adult male RIPK1^{LPC-KO} (deficient for RIPK1 specifically in liver parenchymal cells) C57BL/6 male mice and age-matched wild type (RIPK1$^{fl/fl}$) littermates were given a standard diet (SD) (5001, LabDiet, St. Louis, MO) or a high-fat high cholesterol diet (HFHCD) enriched in cholate, described elsewhere (*28*) ad libitum for 3,5 and12 weeks. All mice were reared in specific pathogen-free conditions at the local animal house facilities. The study was conducted in accordance with French law and institutional guidelines for animal welfare. All efforts were made to minimize suffering and the number of animals involved. The protocol was approved by the "Comité Rennais d'Ethique en matière d'Expérimentation Animale", the local ethics committee accredited by the French Ministry of Research and Higher Education (Saisine No 5656).

Histological, immunohistochemical and biochemical analyses

Liver pathology was characterized by H&E stained sections were evaluated for histopathology of NASH like steatosis, lobular infiltrates and hepatocytes ballooning(*28*). Liver fibrosis was assessed by Sirius red coloration, and the stained area was calculated from the total liver surface using NIS-Elements software (Nikon). Serum biochemical analyses of transaminase levels (AST/ALT) were performed as described previously (*29*).

RNA Isolation and RT-qPCR

For the protocol and conditions of RNA extraction, RT and qPCR were similar as reported earlier by our laboratory(*28*). Briefly, Reverse Transcriptase (Applied Biosystems, #4368813) and real-time quantitative PCR was performed using the the double- strand specific SYBR® Green system (Applied Biosystems, #4367659) on CFX384 TouchTM Real-Time PCR Detection System (Biorad). Each measurement was performed in triplicate. The relative gene expression was normalized against the 18S gene expression.

Total RNA was extracted from mice liver tissues and from primary hepatocytes using the NucleoSpin® RNA kit (Macherey-Nagel, #740955). First-strand cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368813, Foster City, CA, ®USA). Real-time quantitative PCR was performed using the double-strand specific SYBR system (Applied Biosystems, #4367659) on CFX384 Touch^{TM} Real-Time PCR Detection System (Biorad). Each measurement was performed in triplicate. The relative gene expression was normalized against the 18S gene expression. The SD chow fed mice served as reference for mRNA expression (control mRNA level was arbitrarily set at 1).

Serum cytokine immunoassay by flow cytometry

Murine cytokines (IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, MCP-1, IFN-β, IFN-γ, TNF-α, and GM-CSF) were quantified by bead-based immunoassays according to manufacturer protocol, using a filter plate and a vacuum filtration system for washing steps (BioLegend's LEGENDPLEX, multi-analyte flow assay kit). Samples were analyzed on an LSR Fortessa cytometer (BD Biosciences).

Isolation of liver immune cells and flow cytometry

The cells were isolated as descrive previously(*28*). Briefly, the liver immune cells were isolated as previously described, with a viability > 95%. Liver cells were resuspended in staining buffer (10% FCS in PBS) and incubated with anti-CD16/32 antibody (BD Pharmingen) to block non-specific binding. The cells were then labeled with the appropriate fluorochrome-conjugated antibodies/ reagents (BD Pharmingen and eBioscience) according to the manufacturer's instructions. The stained cells were analyzed on a FACSAriaTM II flow cytometer with BD FACSDiva software (BD Bioscience) and the data analyzed using BD FACSDiva software (BD Bioscience). Doublets and dead cells were excluded on the basis of forward/side scatter and LIVE/DEAD labeling, respectively. We calculated the percentage of each immune cell

population, by considering the sum of events of all immune cell populations analyzed (sum of T, NK, NKT, B cells or myeloid cells) as 100% of the total immune cells. The absolute number in each immune cell population was calculated by multiplying the percentage of each population by the total number of immune cells.

Statistical analysis

The results shown are representative of two independent experiments and are expressed as the means ± SEM for each group of mice (3 to 10 mice per group from two independent experiments). We used the nonparametric Mann-Whitney U test, as implemented in GraphPad Prism5 software. Differences were considered significant for $p < 0.05$ and are indicated as follows: * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

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LEGENDS

Figure 1. HFHCD induces liver transcript expression of death ligands and their cognate receptors as well as RIPK1(A) Liver transcript expression of death ligands (TNF-α, FASL, TRAIL) and their cognate receptors (TNFR1, TNFR2, FAS, DR5) in SD and HFHCD fed WT mice (B) Liver transcript expression of RIPK1. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 2. HFHCD feeding resulted in progressive liver damage and hepatomegaly(A) Both *Ripk1*^{fl/fl} and *Ripk1*[LPC-KO] mice were fed HFHCD and SD for 3, 5 and 12 weeks. ALT plasma level and liver to body weight ratio (B) H&E stained liver tissue from both *Ripk1*^{fl/fl} and *Ripk1*[LPC-KO] mice. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 3. HFHCD feeding resulted in significant changes expression of cytokines (A) Liver transcript expression of IL-6, TNF-α and CCL2 (B) Plasma levels of CCL2, TNF-α, IL-10, IL-6, IL-27 and IFN Beta. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 4. Gating strategy of Immune cells. Liver single cells were characterized on the basis of expression of CD19, CD3 and TCRVB as shown in figure.

Figure 5. HFHCD feeding resulted in significant inflammatory changes in both $Ripk1^{fl/fl}$ and $Ripk1^{LPC-KO}$ mice. (A) Number of immune cells per liver (B) Expression of CD69 in LT4, LT8, LB, NK cells and NKT cells. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 6. Deficiency of RIPK1 in liver parenchymal cells sensitized to liver fibrosis. (A) Liver transcript expression of Col1α, αSMA, TIMPs and MMPs in $Ripk1^{fl/fl}$ and $Ripk1^{LPC-KO}$ mice and percentage of serius red positive lower panrel right. (B) Sirius red stained liver tissue issued from $Ripk1^{fl/fl}$ and $Ripk1^{LPC-KO}$ mice. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 7. HFHCD feeding induced oxidative stress (A) Liver transcript expression of Nox-2 Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Fig 1A

Fig 1B

Fig. 2A

Fig. 2B

Ripk1^fl/fl   H&E staining   Ripk1^LPC-KO

Fig. 3A

TNFα

IL-6

CCL2

Fig. 3B

CCL2

TNFα

IL-10

IL-6

IL27

IFN-ß

Fig. 4

Fig. 5A

Hepatic immune cells

Fig. 5B

Fig. 6A

COL1A1

α-SMA

TIMP2

TIMP 1

MMP2

TGFBi

Fig. 6B

Sirius Red staining
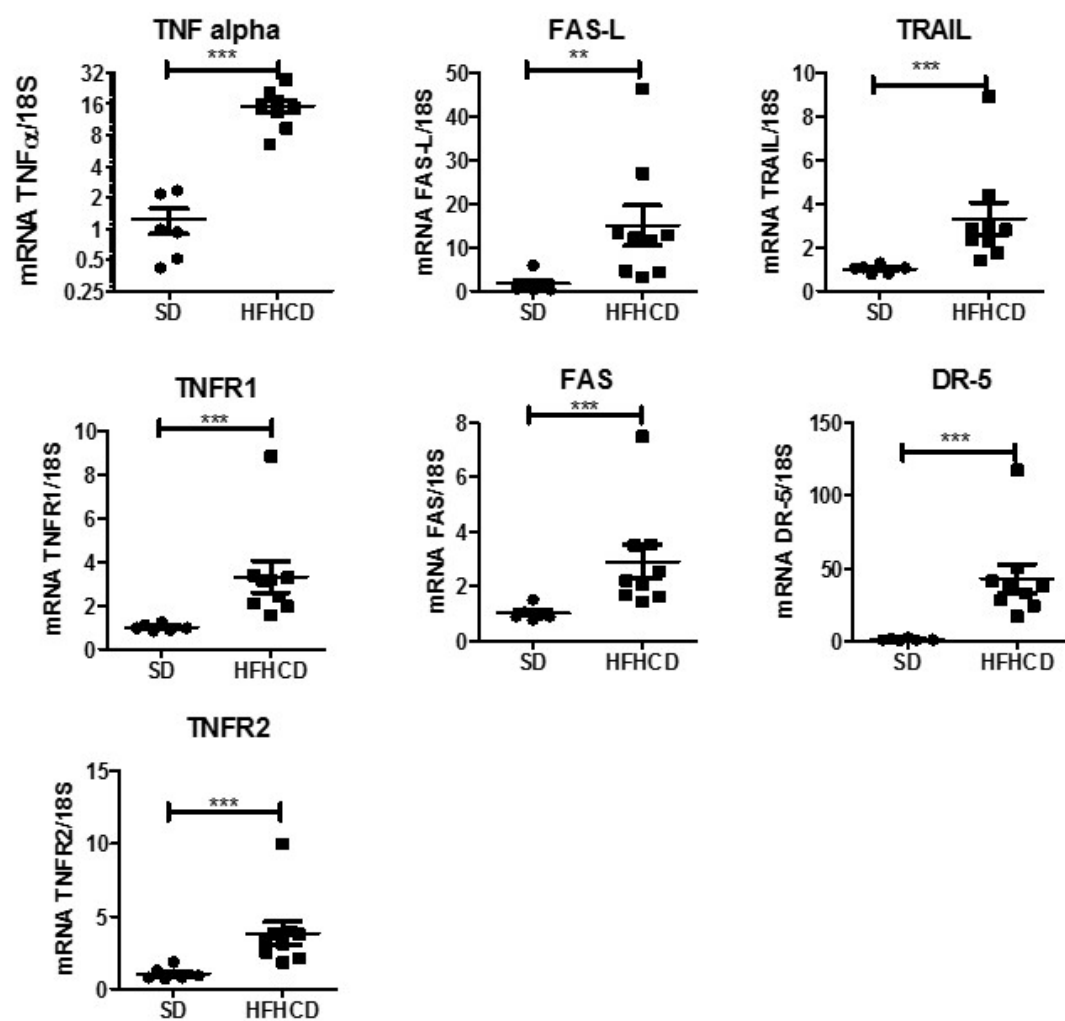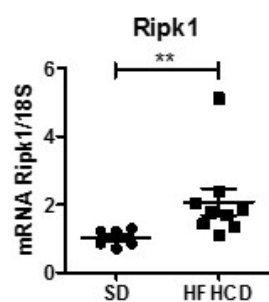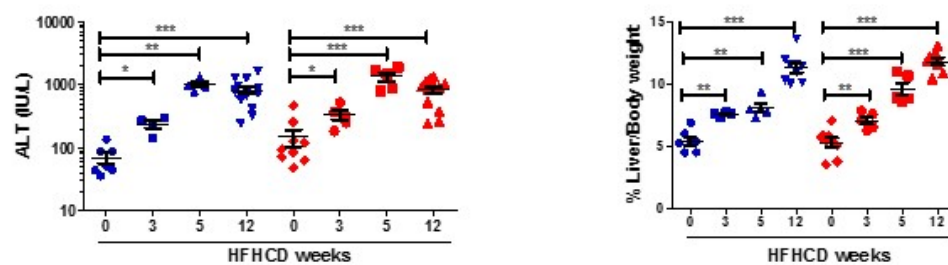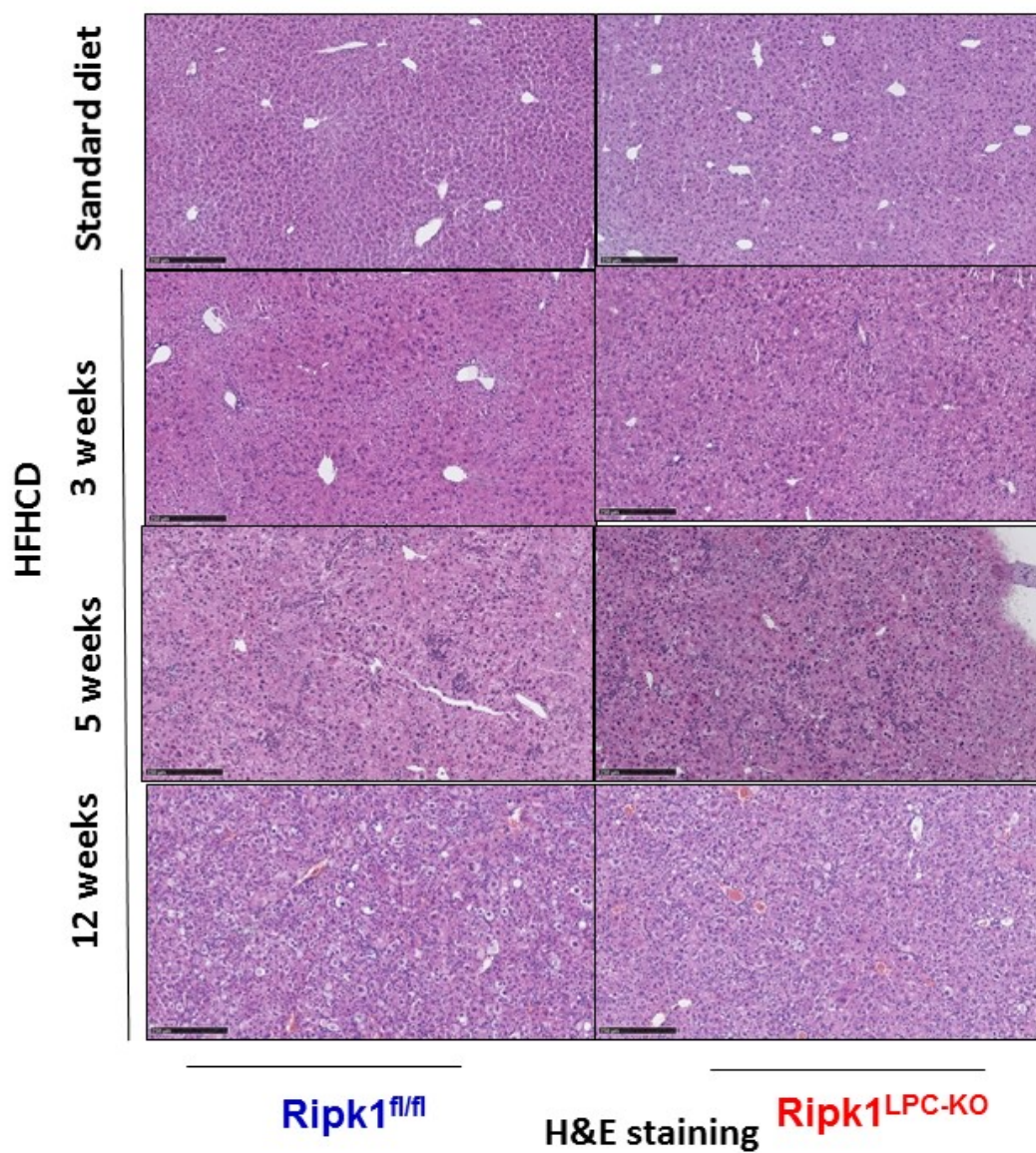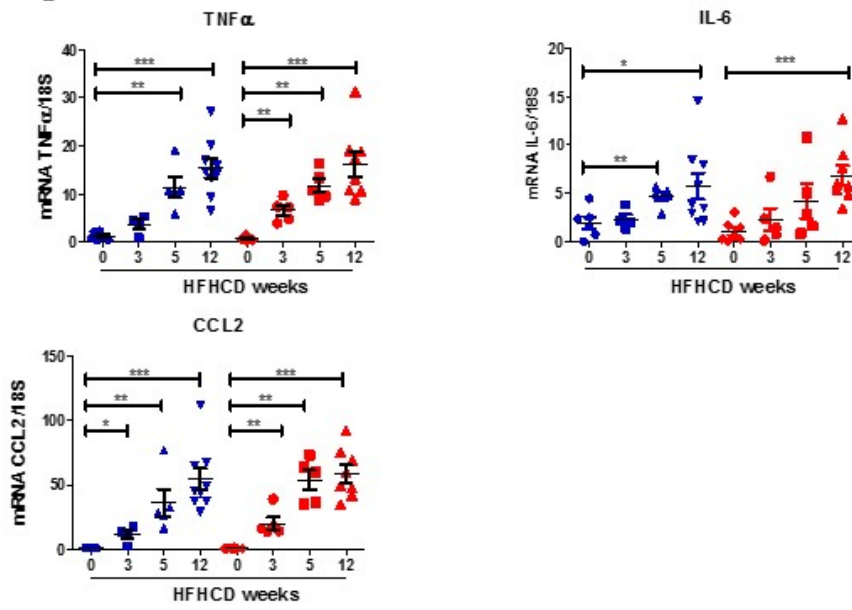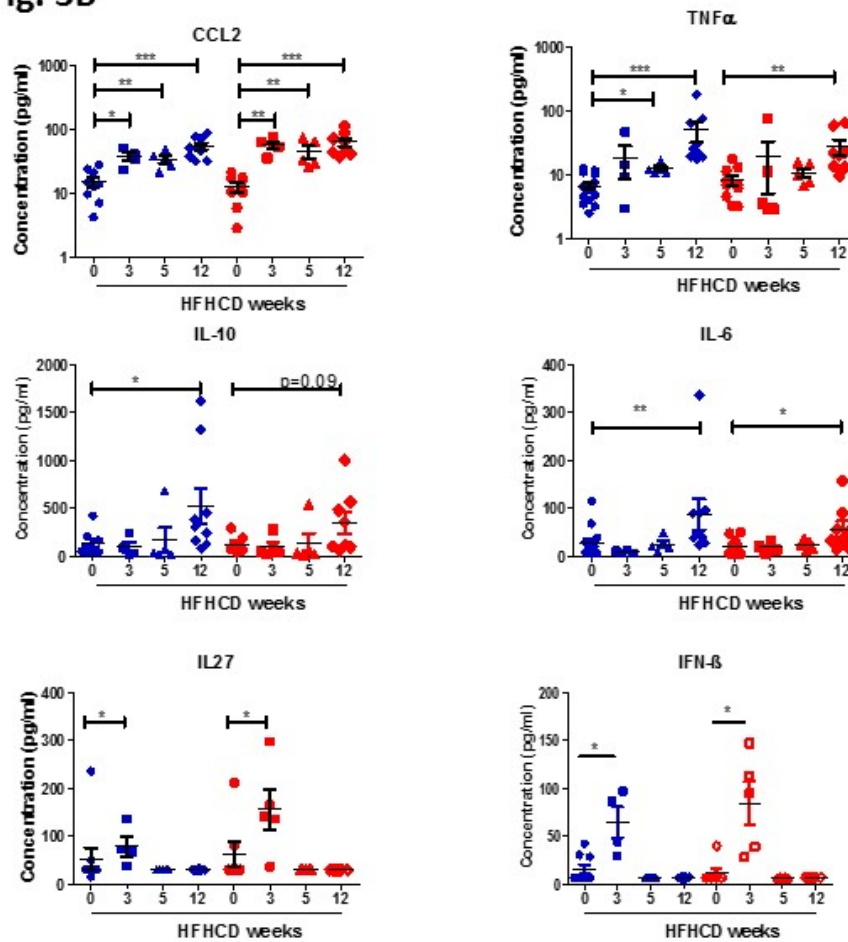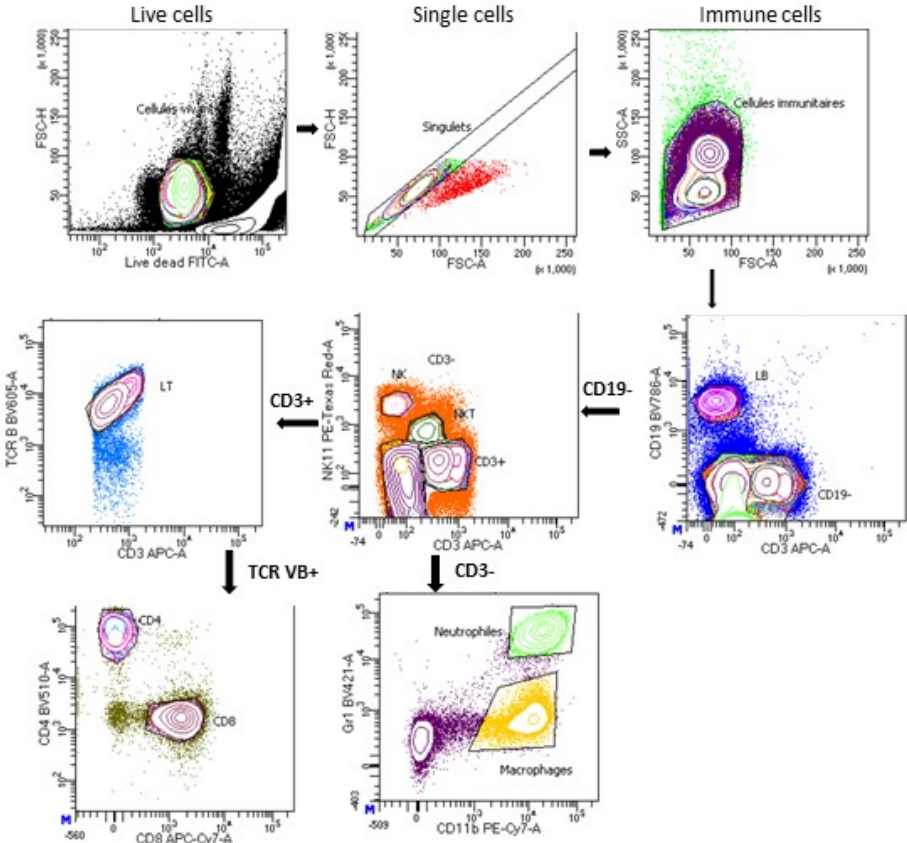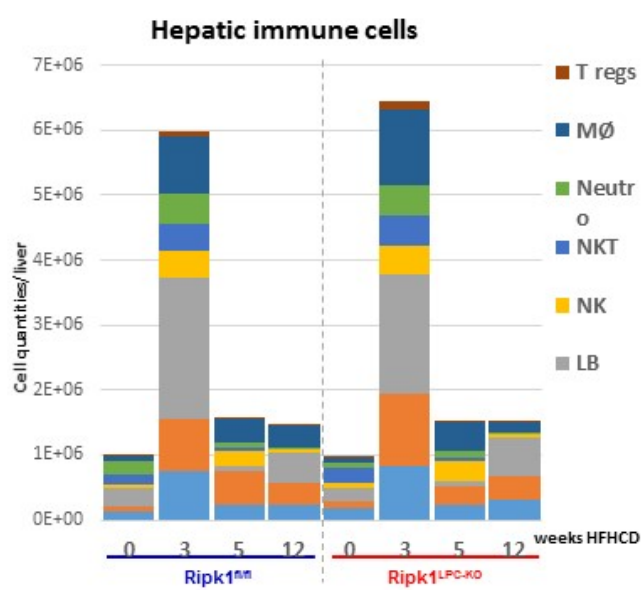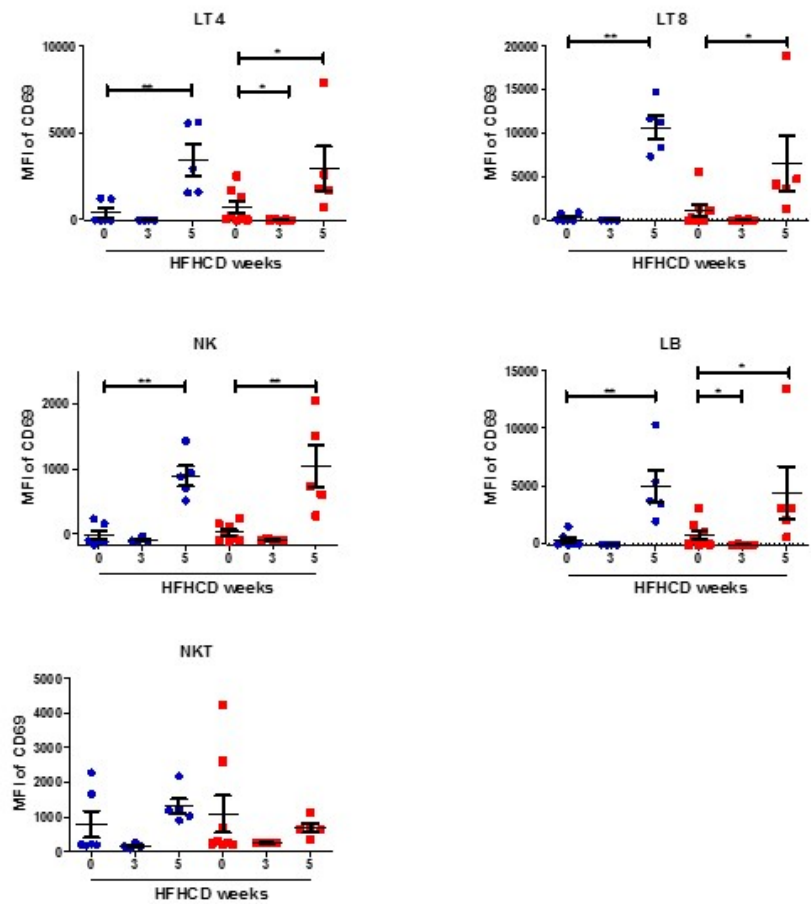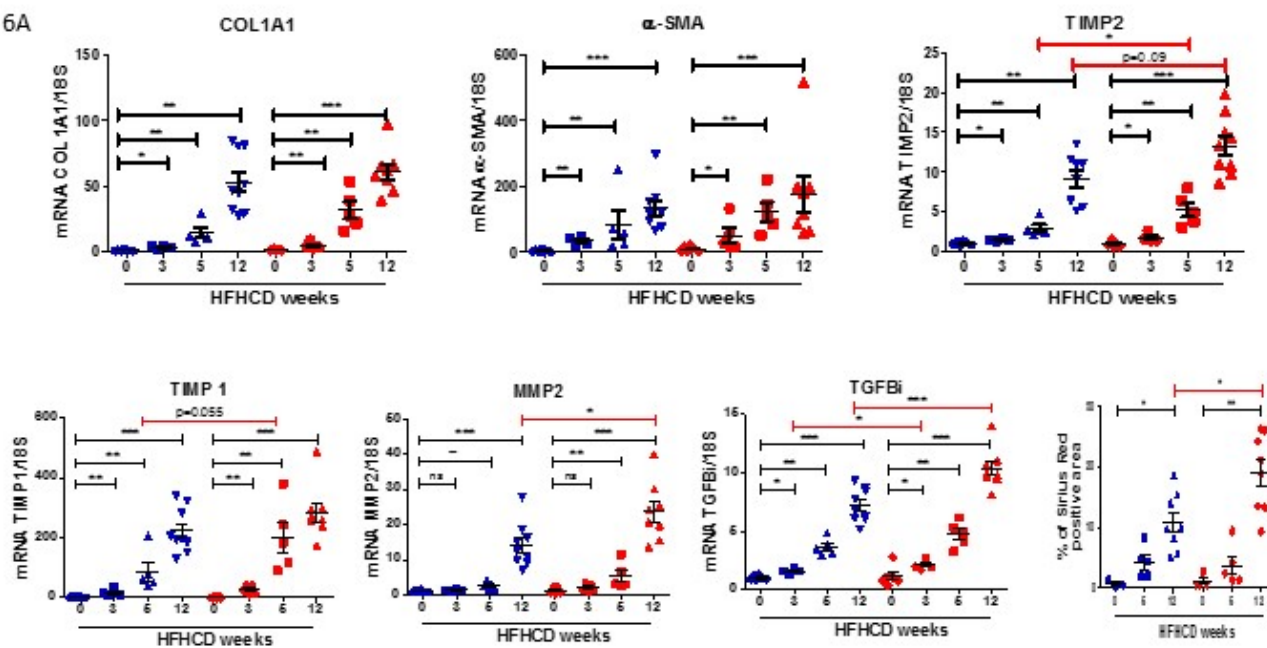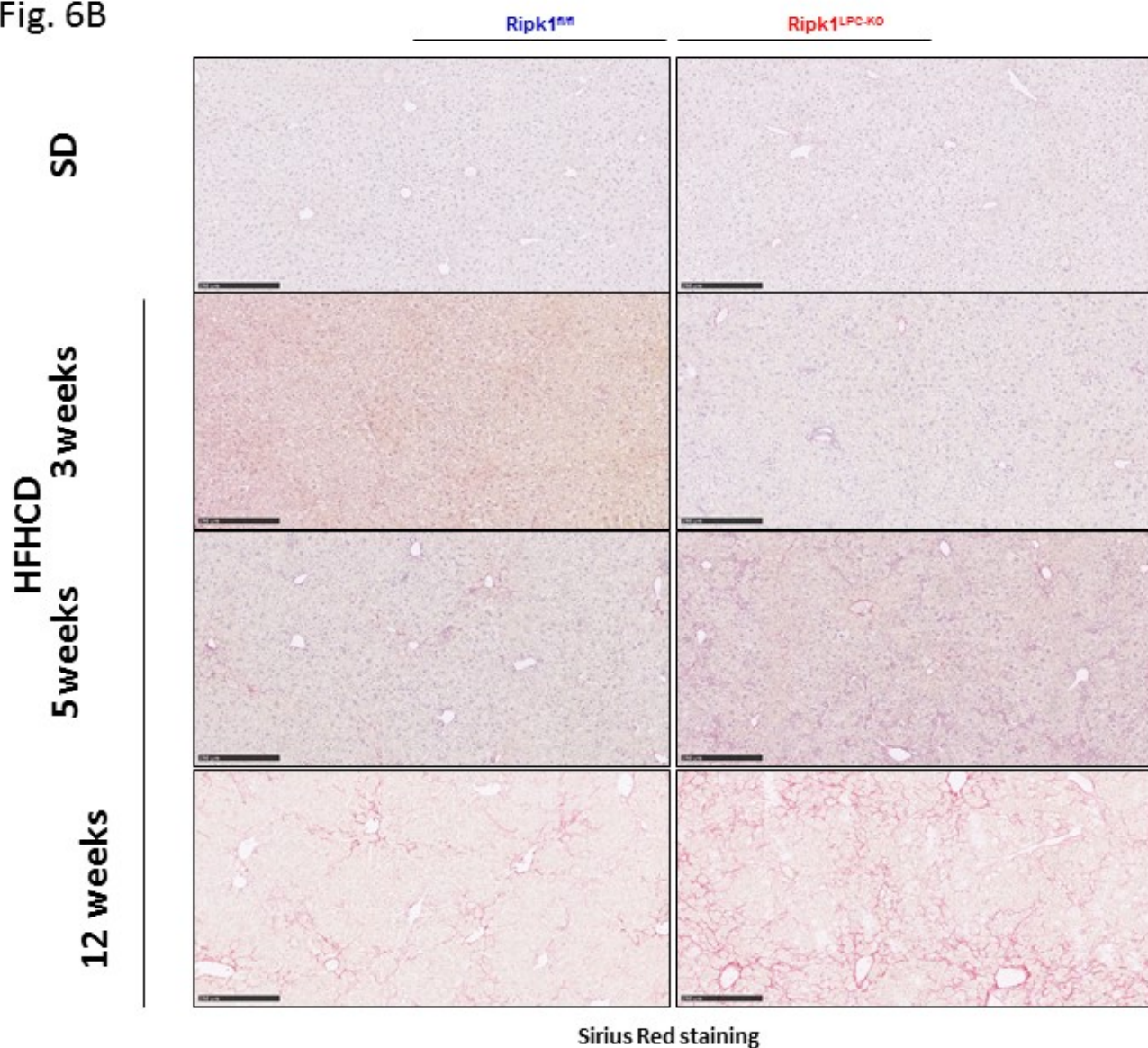
Fig 7
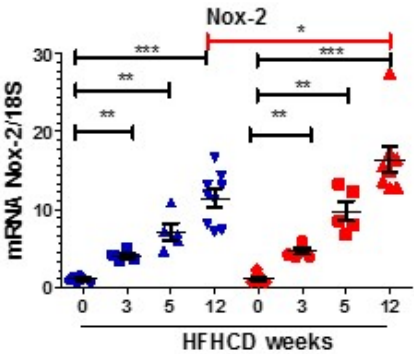
SUPPLEMENTARY TABLE 1 (Reference of antibodies used in FACS)

Antibodies against surface markers	Flurochromes	Clone	Reference	Dilution
CD3	APC	145.2.C11	eBio 17-0031-81	1/100
CD3	PE-CF594	145.2C11	BD 562286	1/150
CD4	PE-Cy7	RM4-5	BD 561099	1/150
CD8	APC- Cy7	53-6-7	BD 557654	1/100
CD19	BV786	ID3	BD 563333	1/100
TCR-VBeta	BV605	H57-597	BD 562840	1/50
NK1.1	PerCP-CY5.5	PK136	eBio 45-5941-82	1/200
NK1.1	PE-eFluoro610	PK136	eBio 61-5941-80	1/100
NK1.1	BV421	PK136	BD 562921	1/50
CD69	PerCP-Cy5.5	H1.2F3	Biolegend 104522	1/25
CD11b	BV510	M1/70	BD 562950	1/200
CD11b	PE-Cy7	M1/70	BD 552850	1/200
F4/80	PE	BM8	eBio 12-4801-82	1/50
LY-6G	APC-H7	1A8	BD 565369	1/100
Grl	Ef450	RB6-8C5	eBio 48-5931-82	1/100

Supplementary Table 2. Sequence of Primers

Gene	Forward	Reverse
Mouse 18S	5′-CGCCGCTAGAGGTGAAATTC-3′	5′-TTGGCAAATGCTTTCGCTC-3′
Mouse TNFα	5′-TAGCTCCCAGAAAAGCAAGC-3′	5′-TTTTCTGGAGGGAGATGTGG-3′
Mouse IL-6	5′-CCGGAGAGGAGACTTCACAG-3′	5′-CAGAATTGCCATTGCACAAC-3′
Mouse TIMP1	5′- TTCCAGTAAGGCCTGTAGC-3′	5′-TTATGACCAGGTCCGAGTT -3′
Mouse Timp2	5′-CTGGGACACGCTTAGCATCA -3′	5′-GACAGCGAGTGATCTTGCAC -3′
Mouse CCL2	5′-TCCCAATGAGTAGGCTGGAG-3′	5′-TCTGGACCCATTCCTTCTTG -3′
Mouse alpha SMA	5′-GGAATCCTGTGAAGCAGCTC -3′	5′- CAGAGCCATTGTCACACACC-3′
Mouse Coll 1 Alpha	5′-GCTCCTGCTCCTCTTAGGG -3′	5′-GCAGAAAGCACAGCACTCG -3′
Mouse MMP 2	5′- ATGGTAAACAAGGCTTCATG-3′	5′-TTGAGAAGGATGGCAAGTAT -3′
Mouse MMP 8	5′- TACAGGGAACCCAGCACCTA-3′	5′-GGGGTTGTCTGAAGGTCCATAG -3′
Mouse MMP 9	5′-CATTCGCGTGGATAAGGAGT -3′	5′-TCACACGCCAGAAGAATTTG-3′
Mouse MMP 13	5′-ACACTGGCAAAAGCCATTTC-3′	5′-TTTTGGGATGCTTAGGGTTG-3′
Mouse MMP 14	5′-GCCCTCTGTCCCAGATAAGC -3′	5′- ACCATCGCTCCTTGAAGACA-3′
Mouse MMP 19	5′- GTGTGGACTGTAACAGATTCAGG-3′	5′-CCTTGAAGAAATGAGTCCGTCGT -3′
Mouse TGFB1	5′-CACCATCCATGACATGAACC -3′	5′- CAGAAGTTGGCATGGTAGCC-3′

Article 4. Dietary intervention partially resolves high-fat high-cholesterol induced liver fibrosis in mice

(Article in preparation)

Background and aims

Non-alcoholic steatohepatitis (NASH) is a world-wide disease, characterized by steatosis, inflammation, liver damage, hepatomegaly with varying level of fibrosis. This hepatic healing state results from the disruption of the delicate balance between extracellular matrix (ECM) deposition and degradation. Fibrosis remains the key predictor of NASH prognosis, and can further progress into cirrhosis and hepatocellular carcinoma. In human, conflicting reports are published on the efficiency of dietary intervention on NASH-advanced fibrosis reversibility. Discrepancies could be due to differences in study design, stage of fibrosis and type of dietary intervention. Besides, effect of dietary intervention on NASH-induced advanced fibrosis in animal models is lacking. Here, we aimed to investigate the effect of dietary intervention in high-fat high-cholesterol diet (HFHCD) induced NASH in mice.

Method

NASH was induced in C57BL6NJ mice by HFHCD feeding for 5 and 12 weeks to respectively induce early non-fibrotic and late fibrotic NASH. To measure the efficiency of dietary intervention in NASH, enrolled mice were further switched to SD for 4 weeks in early non-fibrotic NASH, and for 4 or 12 weeks in late fibrotic NASH. Different clinical-pathological investigations (plasma transaminases measurement, liver histology, fibrosis level) were assessed. Hepatic transcript expression of genes involved in inflammation, oxidative stress and fibrosis was measured by RT-qPCR.

Results:

HFHCD feeding resulted in progressive establishment of steatosis, liver damage, hepatomegaly, oxidative stress in both early non-fibrotic and late fibrotic NASH, as in human. As expected, significant fibrosis only appeared after 12 weeks of HFHCD feeding (named late fibrotic NASH condition). Dietary intervention induced drastic reduction of steatosis, plasma transaminase levels, inflammatory markers (liver transcript expression of TNF and CCL2), hepatomegaly and oxidative stress in both early non-fibrotic and late fibrotic NASH. However, while no significant improvement in fibrosis was detected after 4 weeks of SD in mice previously fed for 12 weeks with HFHCD, a partial reversion was discovered after 12 weeks of SD. Thus, dietary intervention in late fibrotic NASH resulted in drastic decrease in the expression of the matrix metalloproteinases and tissue inhibitors of metalloproteinases, while remaining above the standard chow diet control.

Conclusion:

Our investigations demonstrated that dietary intervention can initiate a reversion process of liver fibrosis in HFHCD-induced NASH.

Dietary intervention partially resolves high fat high cholesterol induced liver fibrosis in mice

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Abstract

Non-alcoholic steatohepatitis (NASH) is world-wide disease, characterized by steatosis, inflammation, liver damage, hepatomegaly with or without fibrosis. Liver fibrosis remains the key predictor of prognosis of NASH. There are conflicting reports on efficiency of dietary intervention on reversibility of advanced fibrosis accompanied by NASH. Here we aimed to investigate the effect of dietary intervention in high fat high cholesterol diet (HFHCD) induced NASH in mice. NASH was induced in C57BL6 mice by HFHCD feeding for 5 and 12 weeks to induce early non-fibrotic and late fibrotic NASH respectively. Different clinic-pathological investigations (plasma transaminases measurement, liver histology, level of fibrosis) were assessed. To measure the efficiency of dietary intervention on histological findings of NASH, diseased mice were further switched to SD for 4 weeks in early non-fibrotic NASH and 4 and 12 weeks in late fibrotic NASH induced mice. HFHCD feeding resulted in progressive steatosis, liver damage, hepatomegaly, oxidative stress in both early non-fibrotic and late fibrotic NASH. However, significant fibrosis was only present in late fibrotic NASH maintained on 12 weeks of HFHCD feeding. Dietary intervention resulted in drastic reduction steatosis, plasma transaminases levels, inflammatory markers (liver transcript expression of TNF and CCL2), hepatomegaly and oxidative stress. However, the liver fibrosis was only partially reversible when mice were switched to SD for 12 weeks only in late fibrotic NASH group. Dietary intervention in late fibrotic NASH, resulted in drastic decrease in the expression of the matrix metalloproteinases and tissue inhibitors of metalloproteinases but still remained higher than standard chow diet control. In conclusion, our results demonstrate that dietary intervention can partially reverse HFHCD feeding induced liver fibrosis.

Introduction

Liver fibrosis is due to a process of deposition of ECM, as a consequence of hepatic damage caused by virus, toxic agent, metabolic syndrome (alcohol or HFD) and bacterial PAMPs absorbed from leaky gut. Chronic persistent liver injury results in activation of hepatic satellite cells (HSCs) leading to deposition of ECM and ultimately liver fibrosis, which can further progress into liver cirrhosis and hepatocellular carcinoma (HCC)[1,2].

In normal liver, there is a balance between synthesis and degradation of ECM. ECM deposition is mainly characterized by deposition of collagen, laminin, proteoglycans, fibronectin or matricellular proteins[3,4]. On the other hand, degradation is regulated by proteases like matrix metalloproteinases (MMPs), a disintegrin and metalloproteinase (ADAM). MMPs activity is further inhibited by tissue inhibitors of MMPs (TIMPs) [3,5]. Disruption of this delicate balance between synthesis and degradation of ECM can result in increased deposition than degradation of ECM results in liver fibrosis.

When the agents causing liver damage disappear, the fibrosis can be reversible, meaning that proteases degrade ECM[6]. However, in some conditions, although fibrosis regresses, it is not fully reversible in patients and can cause severe complication as occurrence of HCC[7-10].

To evaluate the reversibility of liver fibrosis, different mouse models have been used. However, the main model used is chronic CCl4 administration and shows regression of liver fibrosis once exposure of hepatotoxic agent is inhibited [11]. Nevertheless, this model presents a very weak fibrosis compared to human. We develop a HFHCD induced mouse model of NAFLD and we observed until 20% of fibrosis.

Non-alcoholic fatty liver disease (NAFLD) varies from simple steatosis without hepatocellular injury (NAFL) to aggressive form, the non-alcoholic steatohepatitis (NASH) [12]. NAFL is reversible and characterized by at least 5% of liver steatosis. NAFL can progress into NASH,

which is characterized by hepatitis, inflammation, steatosis and cell death with or without liver fibrosis [13]. Non-alcoholic steatohepatitis (NASH) is a serious liver disease characterized by steatosis, liver damage, oxidative stress, elevated transaminases and recruitment of inflammatory cells with or without fibrosis[13]. NASH can further progress into liver cirrhosis and hepatocellular carcinoma. Currently NASH is the 2nd leading cause of liver transplantation and predicted to be the leading cause in next decade[14,15]. NASH is associated with insulin resistance and obesity[16-18].

The pathogenesis of NASH is not fully elucidated. In 2016, Buzzetti et al proposed a hypothesis called multiple-hits; to better understand the mechanism of development of NASH. According to multiple hit hypothesis, lipotoxicity, in parallel with other dietary and genetic factors, adipose tissue dysfunction, and gut microbiome regulate the cell death and inflammation, which leads to development of NASH[19]. Persistent low-grade liver injury results in liver fibrosis which is due to imbalance in ECM deposition and degradation[20].

Human NASH is a complex disease caused by multiple factors and there is no single animal experimental model to mimic wide pathology of human NASH. Previously, it has been demonstrated that dietary intervention in western diet (WD), methionine choline deficient diet (MCD) and choline deficient L-amino acid defined diet (CDAA) results in partial reversibility of NASH [21-23]. All these models have been demonstrated to induce low grade fibrosis and role of dietary intervention in resolving fibrosis remains controversial[21-23]. Beforehand, we have demonstrated that HFHCD feeding in mice induces NASH characterized by hepatocyte ballooning, liver inflammation, elevated transaminases and extensive chicken wire liver fibrosis (a key finding in human liver fibrosis in NASH)[24]. Here we aimed to investigate the reversibility of NASH related histopathological parameters in HFHCD. To test this, C57BL6 mice were fed for 5 and 12 weeks of HFHCD feeding before switching to standard chow diet.

Materials and Methods

Animals treatment protocols

Adult C57BL/6 male mice were given a standard diet (SD) (5001, LabDiet, St. Louis, MO) or a high-fat diet (HFD) enriched in cholate, described elsewhere ad libitum for 5 and12 weeks.

Mice were fed HFHCD for 5 weeks and 12 weeks to induce NAFLD. Additionally, mice were fed HFHCD for 5 weeks and maintained for 4 weeks on SD thereafter. Similarly, mice were fed HFHCD for 12 weeks and were further maintained on SD for 4 and 12 weeks.

All mice were reared in specific pathogen-free conditions at the local animal house facilities. The study was conducted in accordance with French law and institutional guidelines for animal welfare.

Histopathological and biochemical studies

Mouse liver fragments were fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemistry. For histopathology, haematoxylin and eosin (H&E) staining of liver tissues was carried out to investigate the liver injury. Liver fibrosis was assessed by Sirius red coloration. Plasma alanine (ALT) and aspartate (AST) transaminases were measured according to the IFCC primary reference procedures using Olympus AU2700 Auto- analyser[R] (Olympus Optical, Tokyo, Japan).

RNA isolation and RT-qPCR

Total RNA was extracted from mice liver tissues and from primary hepatocytes using the NucleoSpin® RNA kit (Macherey-Nagel, #740955). First-strand cDNA was synthesized using

the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, #4368813, Foster City, CA, USA). Real-time quantitative PCR was performed using the double-strand specific SYBR® Green system (Applied Biosystems, #4367659) on CFX384 Touch™ Real-Time PCR Detection System (Biorad). Each measurement was performed in triplicate. The relative gene expression was normalized against the 18S gene expression. The SD fed mice served as reference for mRNA expression (control mRNA level was arbitrarily set at 1). The primer sequences are all depicted in Table S1 in supporting information.

Statistical analysis

Data was expressed as means ± SEM for all mice treated similarly. Mean differences between experimental groups were assessed using the non-parametric Mann–Whitney U-test. All statistical analysis were achieved with the GraphPad Prism5 software. Significance is shown as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Results

HFHCD induced liver damage and hepatomegaly are reversible after switching to SD

We have previously demonstrated that high fat high cholesterol diet (HFHCD) feeding induced NASH in mice mimicking human NASH. Consequently, in order to induce NASH, mice were fed HFHCD for 5 weeks and 12 weeks and were compared to SD control systematically. The HFHCD feeding developed characteristic findings of NASH that is hepatomegaly, hepatocyte ballooning, infiltration of inflammatory cells, increased liver damage. Therefore, HFHCD feeding resulted in progressive liver damage as evidenced by increase in ALT (Fig 1B). However, the level of ALT returned to basal after switching of mice to SD in group "HFHCD 5 weeks + SD 4 weeks", group "HFHCD 12 weeks + SD 4 weeks" and group "HFHCD 12 weeks + SD 12 weeks". Similarly, HFHCD feeding induced progressive hepatomegaly, which returned to basal level, when mice were switched to SD.

Additionally, HFHCD feeding resulted in hepatocyte ballooning and recruitment of inflammatory cells as evidenced by H&E stained tissues (Figure 2A, left panel). Furthermore, immune cells infiltrates diminished significantly in group when mice were switched to SD. Similarly, expression of Liver transcripts of inflammatory markers like TNF-α and CCL2 increased significantly at 5 weeks post HFHCD feeding and continue to rise till 12 weeks post HFHCD feeding. However, switching to SD resulted in return of liver transcript expression of both TNF-α and CCL2 to basal level (Figure 3A). Oxidative stress also plays key role in the pathogenesis of NASH. Next, we determined the expression of liver transcripts of genes like Nqo1 and Nfr2 involved in oxidative stress. Here we demonstrated that HFHCD feeding resulted in significant increased expression of both Nqo1 and Nfr2 in time dependent manner, which returned to basal level, when mice were switched to SD.

HFHCD induced fibrosis is partially reversible

Liver fibrosis in NASH results from chronic persistent liver injury induced deposition of ECM. Next, we aimed to determine the level of fibrosis. HFHCD feeding for 5 weeks did not revealed significant liver fibrosis as compared to SD fed mice. In contrast HFHCD feeding for 12 weeks resulted in significant increase in liver fibrosis as evidenced by sirius red positive area (Figure 2A right panel). After 12 weeks of HFHCD feeding, switching to SD for 4 and 12 weeks resulted in no and partial regression of liver fibrosis respectively (Figure 2B). Next, we measured liver transcript expression of proteins involved in a balance of liver deposition or degradation of ECM. Expression of liver transcript of Alpha SMA and Coll 1 alpha, good indicators of deposition of ECM, were significantly higher at 5 weeks post HFHCD feeding and continue to increase at 12 weeks post HFHCD feeding. Furthermore, expression of liver transcript of these proteins returned to base level in all groups after switching to SD.

Consequently, we chose to explore the liver transcript expression of MMPs and TIMPs. HFHCD feeding resulted in increased expression of liver transcripts of MMP-2 and MMP-13. However, expression of liver transcripts of MMP-2 remained at basal level after 5 weeks of HFHCD feeding. Switching to SD resulted in significant decrease in expression of MMP-2 and MMP-13. However, its expression remained slightly higher when mice were switched to SD after 12 weeks of HFHCD feeding. HFHCD feeding also resulted in progressive increase in expression of TIMP-1 and TIMP-2 at both 5 and 12 weeks post HFHCD feeding while TIMP-3 expression was only significantly upregulated at 12 weeks HFHCD feeding. The expression of all TIMPs returned to base level in "HFHCD 5 weeks + SD 4 weeks" group while decreased but remains higher than basal level in "HFHCD 12 weeks + SD 4 weeks" and "HFHCD 12 weeks + SD 12 weeks" treated mice.

Discussion

Liver fibrosis results in response to persistent chronic liver injury. In healthy liver, there is balance between ECM deposition and degradation. Increased deposition through activated HSCs or decrease in degradation results in liver fibrosis. Liver ECM is degraded by MMPs which are inhibited by TIMPs[25,26]. Effect of dietary intervention on advanced fibrosis in diet induced NASH is not well characterized. Currently, there is no FDA approved treatment for NASH[27,28]. In human epidemiological studies show that dietary changes and exercise ameliorates histological parameters like steatosis and ballooning in NASH[29,30]. However, there are conflicting reports on effect of dietary intervention or exercise and bariatric surgery on reversibility of fibrosis in NASH patients[2,8,9,30]. Liver fibrosis remains the strongest predictor of mortality in NASH patients[31,32]. Data on reversibility of advanced fibrosis in mice models of NASH is lacking. Here we demonstrated that HFHCD feeding for 12 weeks resulted in NASH with significant fibrosis.

In HFHCD induced NASH, we have revealed that switching to standard chow diet for 4 weeks in early non-fibrotic NASH resulted in significant amelioration in histological findings of NASH. While in advanced fibrotic NASH, dietary intervention resulted in amelioration of liver steatosis, inflammation and oxidative stress. Inflammation and oxidative stress, key characteristics in NASH, play a key role in the progression of NASH[33]. In accordance with other models (methionine choline deficient diet, choline deficient L-amino acid defined diet, and Western diet) of NASH, we demonstrate that switching to chow diet in both early non-fibrotic and late fibrotic NASH, result in improvement in inflammation and oxidative stress[21,22,34]. Additionally, we have also demonstrated that SD feeding resulted in cessation of new liver damage as demonstrated by basal level of transaminases and diminished hepatomegaly (Figure 1A).

HFD feeding resulted in NASH without fibrosis but addition of cholesterol resulted in significant increase in liver inflammation and fibrosis. In human, both obese and non-obese NASH patients have higher level of plasma cholesterol as compared to healthy control[35,36]. However, partial reversion of liver fibrosis in late fibrotic NASH was only attained when mice were maintained under chow diet for 12 weeks. In contrast, liver fibrosis induced by chronic administration of CCl4 and alcohol in rodents resolved completely within 4-7 weeks, when CCl4 and alcohol exposure was withdrawn[1,11,37-39]. However, in our model of late fibrotic NASH, dietary intervention resulted in amelioration of liver fibrosis and resulted in decrease in TIMPs and MMPs which are still higher than basal expression. Anti-TIMP1 antibodies resulted in improvement in CCl4 induced liver fibrosis in rodents[40]. In contrast, another independent study shows persistent liver fibrosis in CDAA induced NASH and dietary intervention group after 7 weeks of recovery period[22]. This discrepancy could be due to specie variation, stage of liver fibrosis, difference in diet composition and duration of dietary intervention. Further studies with longer dietary intervention will be required to resolve this discrepancy.

In conclusion our results demonstrate that dietary intervention resulted in amelioration of hepatomegaly, cessation of liver damage, decreased oxidative stress and inflammation in both fibrotic and non-fibrotic NASH. However, dietary intervention resulted in partial regression in liver fibrosis in fibrotic NASH. Therefore, therapeutic potential of dietary intervention in combination with anti-fibrotic agents needs to be explored.

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Legends of figures

Figure 1. Dietary intervention resulted in significant changes in hepatomegaly and liver damage (A) Percentage of liver weight to body weight in HFD fed groups and HFD fed group to intervention group (switched to SD) compared to SD fed mice. (B) Level of ALT in plasma. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and ns= non-significant).

Figure 2. Dietary intervention reduced advanced NASH induced fibrosis (A) H and E stained liver sections (Left panel) and Sirius Red stained liver sections (Right Panel). (B) Percentage of Sirius red stained positive area. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 3. Dietary intervention significantly improved liver transcript expression of genes involved in inflammation and ECM deposition (A) Liver transcript expression of TNF and CCL2. (B) Liver transcript expression of Tgfb1 and TGFBi. (C) Liver transcript expression of Col1a1 and alpha SMA. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 4. Liver transcript expression of TIMPs and MMPs decreased significantly after dietary intervention (A) Liver transcript expression of Timp-1, Timp-2 and Timp-3. (B) Liver transcript expression of Mmp-2 and Mmp-13. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Figure 5. Dietary intervention reduced expression of genes involved in oxidative stress (A) Liver transcript expression of Nqo1 and Nfr-2. Statistical analysis was performed by applying non-parametric Mann-Whitney test (*p<0.05, ** p<0.01, *** p<0.001 and ns= non-significant).

Fig 1

A

B

Fig 2

A

H&E   Sirius Red

SD   250μm   500μm

HFHCD 5 W   250μm   500μm

HFHCD 5 W+ SD 4 W   250μm   500μm

HFHCD 12 W   250μm   500μm

HFHCD 12 W+ SD 4 W   250μm   500μm

HFHCD 12 W+SD 12 W   250μm   500μm

B

Fig 3

A

B

C

Fig 4

A

Timp-1

TIMP-2

Timp-3

B

mmp-2

mmp-13
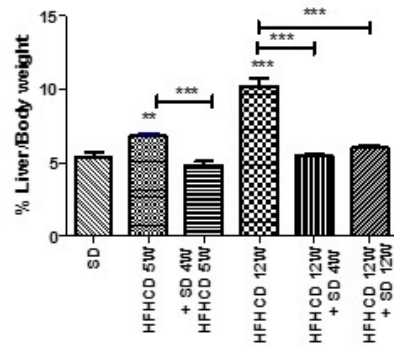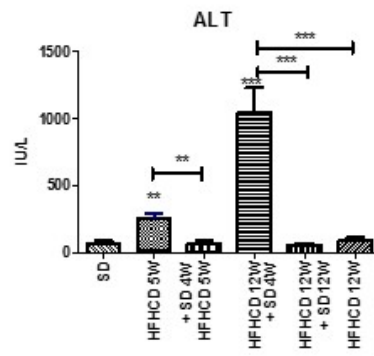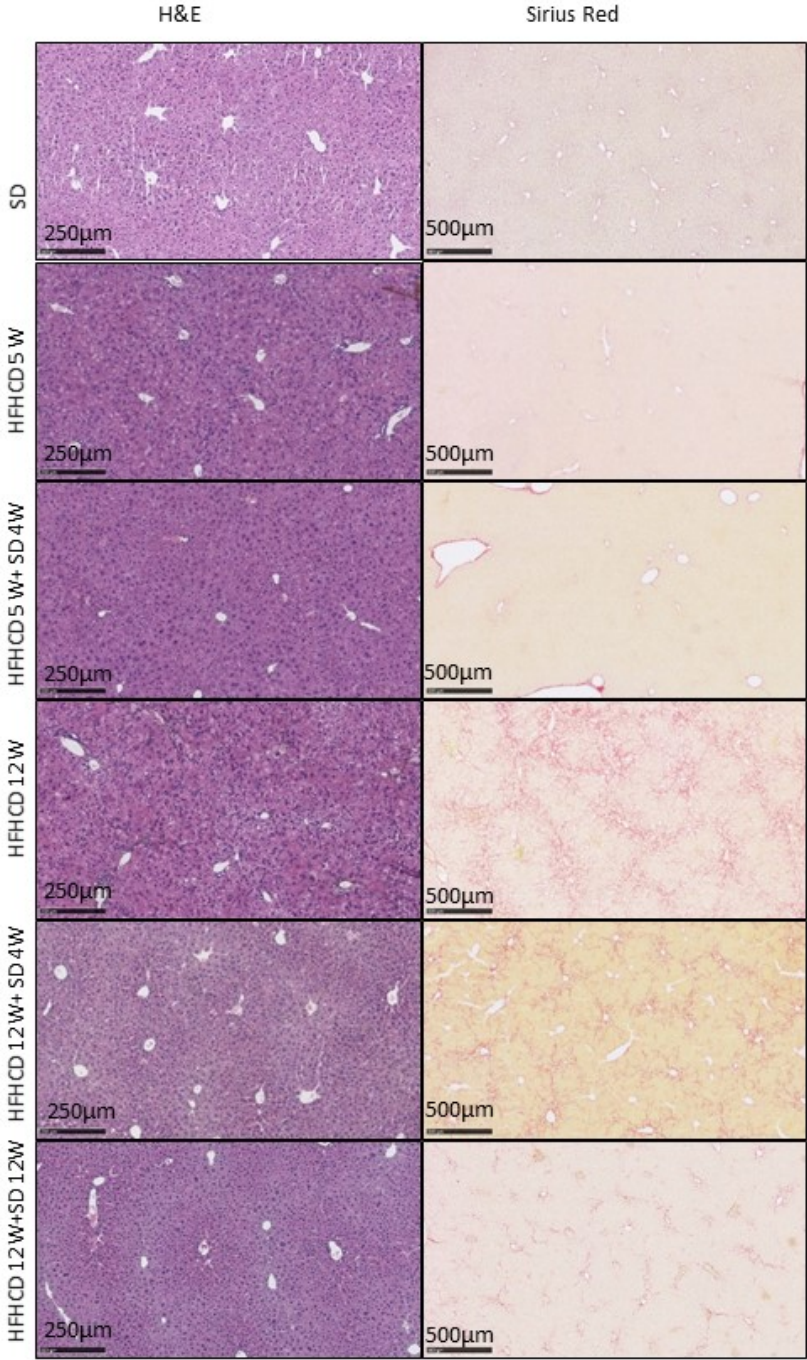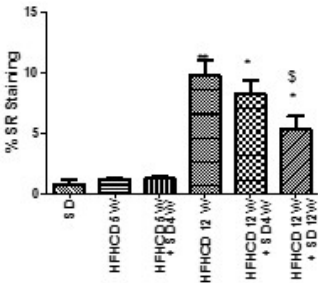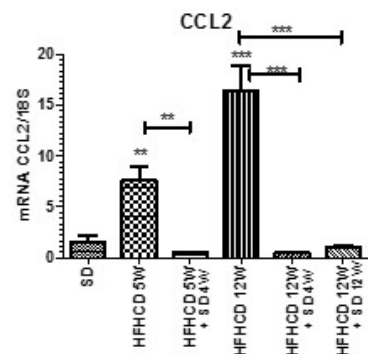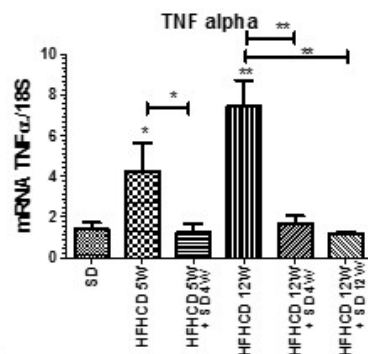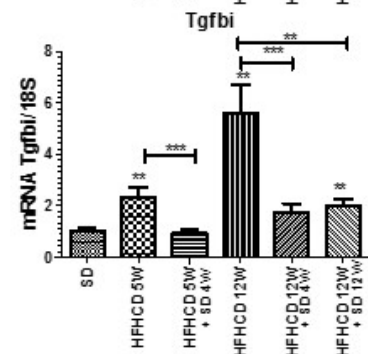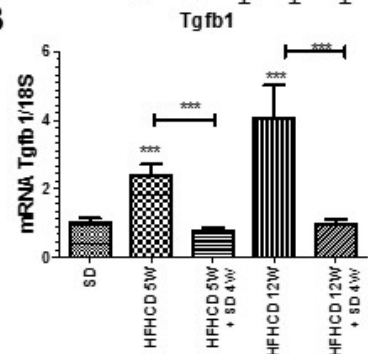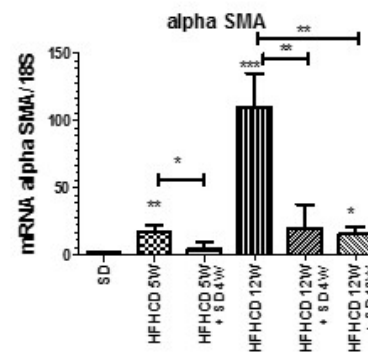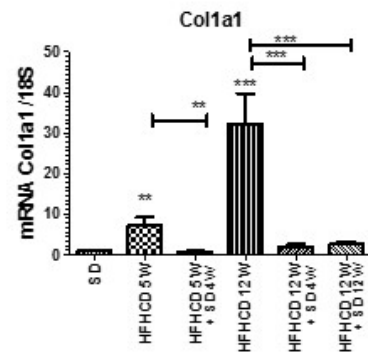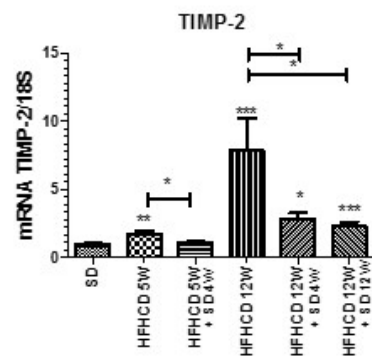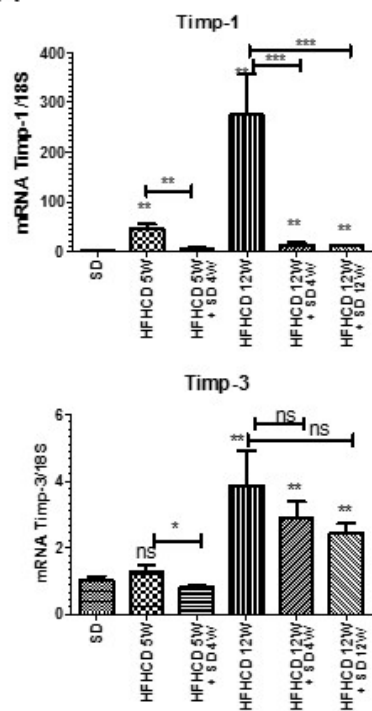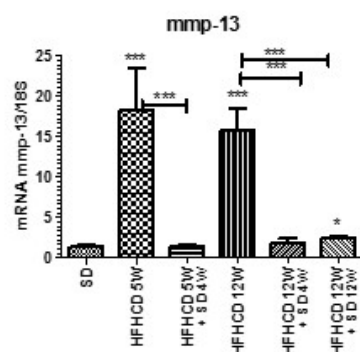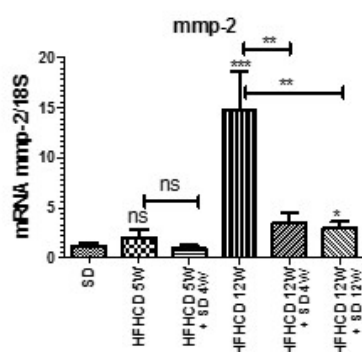
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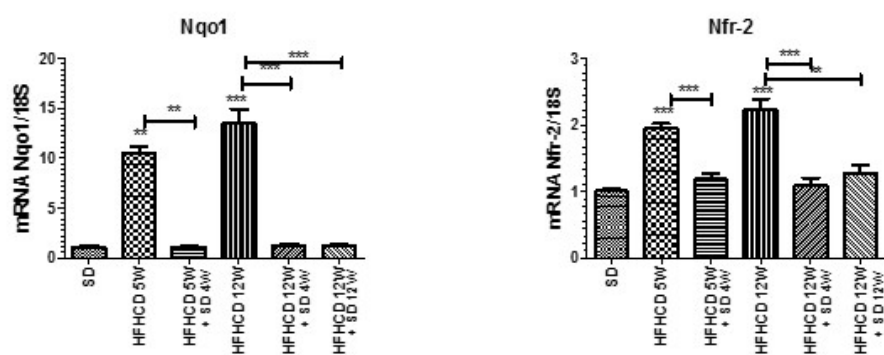
Supplementary Table 1

Sequence of Primers

Gene	Forward	Reverse
Mouse 18S	5'-CGCCGCTAGAGGTGAAATTC-3'	5'-TTGGCAAATGCTTTCGCTC-3'
Mouse TNFα	5'-TAGCTCCCAGAAAAGCAAGC-3'	5'-TTTTCTGGAGGGAGATGTGG-3'
Mouse IL-6	5'-CCGGAGAGGAGACTTCACAG-3'	5'-CAGAATTGCCATTGCACAAC-3'
Mouse TIMP1	5'- TTCCAGTAAGGCCTGTAGC-3'	5'-TTATGACCAGGTCCGAGTT -3'
Mouse Timp2	5'-CTGGGACACGCTTAGCATCA -3'	5'-GACAGCGAGTGATCTTGCAC -3'
Mouse CCL2	5'-TCCCAATGAGTAGGCTGGAG-3'	5'-TCTGGACCCATTCCTTCTTG -3'
Mouse alpha SMA	5'-GGAATCCTGTGAAGCAGCTC -3'	5'- CAGAGCCATTGTCACACACC-3'
Mouse Coll 1 Alpha	5'-GCTCCTGCTCCTCTTAGGG -3'	5'-GCAGAAAGCACAGCACTCG -3'
Mouse MMP 2	5'- ATGGTAAACAAGGCTTCATG-3'	5'-TTGAGAAGGATGGCAAGTAT -3'
Mouse MMP 8	5'- TACAGGGAACCCAGCACCTA-3'	5'-GGGGTTGTCTGAAGGTCCATAG -3'
Mouse MMP 9	5'-CATTCGCGTGGATAAGGAGT -3'	5'-TCACACGCCAGAAGAATTTG-3'
Mouse MMP 13	5'-ACACTGGCAAAAGCCATTTC-3'	5'-TTTTGGGATGCTTAGGGTTG-3'
Mouse MMP 14	5'-GCCCTCTGTCCCAGATAAGC -3'	5'- ACCATCGCTCCTTGAAGACA-3'
Mouse MMP 19	5'- GTGTGGACTGTAACAGATTCAGG-3'	5'-CCTTGAAGAAATGAGTCCGTCGT -3'
Mouse TGFB1	5'-CACCATCCATGACATGAACC -3'	5'- CAGAAGTTGGCATGGTAGCC-3'

IV. DISCUSSION AND PERSPECTIVES

Cell death plays central role in the development and progression of liver diseases (32, 61). Under steady state conditions, there is very little cell death and is generally non-reactive. However, cell insult, due to viral, bacterial, toxic or autoimmune causes, results in hepatocyte destruction, leading to inflammation and compensatory proliferation. In addition, the persistent cell demise, in association with inflammation induced by damage associated molecular pattern or apoptotic bodies, can lead into fibrosis and ultimately hepatocellular carcinoma (32, 61), the 3rd leading cause of cancer related death (269). Irrespective of etiological agent, expression or release of death ligands, such as TNFα, FAS L and TRAIL, by inflammatory cells remains the key players in the progression of liver diseases. Cell death could occur in different ways, such as apoptosis and necroptosis, and its inhibition can be of therapeutic value (32, 61, 270).

Cytosolic kinase RIPK1 influences the fate of cell, whether to survive or to die by caspase-dependent apoptosis or by RIPK3/MLKL-dependent necroptosis and could therefore be a potential target in regulating cell death. This kinase plays key role in response to various death stimuli, like death ligands (TNFα, FAS L and TRAIL), PAMPs (bacterial LPS and viral dsRNA), interferons, DNA damage, stresses including but not limited to calcium overload, endoplasmic reticulum stress (153, 198-205). *Ripk1-/-* mice (deficient for RIPK1 ubiquitously), died within 3 days of their life. Even if these mice were apparently normal without any gross lesions, histological examination revealed massive cell death in lymphoid, intestinal as well as adipose tissue suggesting the key role of RIPK1 in homeostasis of these tissues (247, 248). Further analysis revealed NF-κB defective signalling in *Ripk1* deficient cells downstream of TNFR1 signalling (248). Additionally, RIPK1 kinase dead knock in mice (*Ripk1KD*) were normal suggesting the kinase independent function of RIPK1 in survival. Therefore, kinase independent function(s) of RIPK1 is or are responsible for the early mortality of *Ripk1-/-* mice (249). Unlike *Ripk1KD* mice, *Ripk1* RHIM deficient mice die within the first week of life, further underlying the kinase-independent role of RIPK1 in homeostasis (250, 251). Taken together,

these studies demonstrate that RIPK1 plays RIPK1 kinase dependent and RIPK1 kinase independent scaffolding, pro-death and pro-survival roles respectively. Combined inhibition of apoptosis and necroptosis through different genetic modifications like *Ripk3* and *Fadd* or *Ripk3* and caspase-8 in *Ripk1-/-* mice resulted in prolonged survival, suggesting the possible involvement of both apoptosis and necroptosis in inducing mortality in *Ripk1-/-* mice (247, 252). These studies strongly suggest protective role of RIPK1 in homeostasis.

Conditional knock out mice deficient for *Ripk1* in intestinal epithelium (*Ripk^{IEC-KO}*), epidermal tissue (*Ripk1^{E-KO}*), hematopoietic cells and dendritic cells sensitize to cell death under steady state condition (253-255, 271). In contrast, mice deficient for *Ripk1* specifically in liver parenchymal cells develop normally without any liver pathology demonstrating that RIPK1 is dispensable in liver homeostasis (167, 256, 257). In conclusion, development of conditional *Ripk1* knockout mice demonstrated its unique tissue specific role in regulating cell survival and death.

Interestingly, in contrast to *in vivo* data, primary mouse hepatocytes harvested from *Ripk1^{LPC-KO}* mice died spontaneously, which is rescued completely by pre-treatment of Zvad-fmk and mainly but not completely by ETA (TNF-α decoy receptor) (166, 167, 257, 259, 272). This demonstrates that RIPK1 deficiency leads to apoptosis in RIPK1-deficient PMH mainly in TNF-α dependent manner. The role of TNF-α in spontaneous death is further supported by the fact that additional depletion of TNFR1 in RIPK1 deficient PMH largely protected from apoptosis (257). This TNF-α could be due to autocrine production or released by contaminating immune cells. In contrast, Suda, *et al.* did not observe spontaneous PMH demise harvested from ASO depleted RIPK1 knock down mice (258). This could be due to very low seeding density of PMH or inefficient complete depletion of RIPK1. Neutralization of FASL did not improve observed apoptosis in RIPK1-deficient primary hepatocytes demonstrating that FASL did not

contribute to their apoptotic death (257). However, it remains to be elucidated what causes additional TNF-α-independent apoptosis in RIPK1-deficient primary hepatocytes.

Previously, we and others have demonstrated that inhibition or depletion of RIPK1 kinase activity protects against apoptosis in ConA-induced hepatitis, while depletion of RIPK1 in liver parenchymal cells sensitizes (167, 258). Moreover, we have already demonstrated the protective role played by RIPK1 in liver parenchymal cells in death ligands- (TNF-α, anti-FAS antibody JO2 and TRAIL+IFN gamma) induced apoptosis. RIPK1 stabilizes TRAF2 and cIAPs to protect from TNF-α-induced cell death. RIPK1 and TRAF2 seems to be compensatory for each other as deficiency of either molecule is dispensable for liver homeostasis, while combined ablation resulted in spontaneous HCC in mice due to spontaneous cell death and continuous regeneration (256). Decreased expression of RIPK1 and TRAF2 in HCC were associated with poor prognosis (256). However, in DEN induced HCC, depletion of RIPK1 in liver parenchymal cells decreased the number of tumors in liver by sensitizing tumor cells to TNF-α induced apoptosis. However, depletion of RIPK1 kinase activity did not improve DEN induced HCC (257). In contrast, depletion of RIPK1 kinase activity resulted in inhibition of HCC in mice deficient for NEMO in liver parenchymal cells by inhibiting apoptosis (216, 273). Future studies will be required to address the discrepancies. Besides, the protective role of RIPK1 was also demonstrated in LPS induced hepatitis. We have demonstrated that LPS administration in mice results in release and expression of TNF-α by kupffer cells that is responsible for increased apoptosis in *Ripk1^{LPC-KO}* mice. In addition, pre-treatment of Nec-1 ameliorated LPS/D-Gal induced liver damage demonstrating the involvement of pro-death function of RIPK1 kinase activity (45). However, data with more specific inhibitor like Nec-1s is lacking in this model.

Concerning the role of RIPK1 in fulminant viral hepatitis, nothing was known. Therefore, in article 1, we aimed to explore the role of RIPK1 in mouse model of viral fulminant hepatitis induced by MHV3 administration. MHV3 inoculation resulted in progressive increase in infection marker, inflammatory meditators like TNF-α and CCL2, irrespective of the genotype. Although all animals died at day 4 post-infection, $Ripk1^{LPC-KO}$ mice remains more sensitive at earlier time point as evidenced by increased plasma transaminases and cleaved caspase-3 stained area. It is quite possible that more differences should have been obtained with inoculation using less viruses. This increased sensitivity in $Ripk1^{LPC-KO}$ could be explained by the increased sensitivity of RIPK1 deficient mice to TNF-α and FASL (258, 274, 275). Similarly, depletion of TNFR1/TNF-α or neutralization of FASL in mice decreased the sensitivity to MHV3 induced liver damage (276-278). Collectively, these results demonstrate that RIPK1 deficiency sensitizes to MHV3 induced liver damage. However, role of RIPK1 kinase activity in MHV3-induced liver damage remains to be explored.

To understand mechanism involved in increased cell demise in $Ripk1^{LPC-KO}$ mice, we further used surrogate model induced by synthetic double stranded RNA (poly I:C) mimicking replicative forms of viral genomes. Usually, to elicit a TLR3-regulated fulminant viral hepatitis, poly I:C should be used in combination with D-galactosamine (D-GalN) (152), a liver-specific transcriptional inhibitor (279). Here we demonstrated that administration of poly I:C alone resulted in increased liver damage in $Ripk1^{LPC-KO}$ mice, while transaminases remains at basal level in control littermates ($Ripk1^{fl-fl}$). Previous studies demonstrated that depletion of RIPK1 in certain cell types such as mouse embryonic fibroblasts or primary human fibroblasts sensitizes mice to RIPK3/MLKL dependent necroptosis. In contrast, it never induced direct or sensitized to TNF-α induced cell death (57, 247). This discrepancy could probably be explained by the limited expression of RIPK3 in hepatocytes (46, 50). Pre-treatment of ETA, efficiently inhibited poly I:C induced liver damage demonstrating the potential role of TNF-α.

Furthermore, we have demonstrated that prior depletion of macrophages ameliorated liver injury demonstrating that TNF-α in this context was most probably released/expressed by kupffer cells (KCs) downstream of TLR3 stimulation. Taken together, this data demonstrates the protective role of RIPK1 in TLR3 induced hepatitis in TNF-α dependent manner which is most probably released or expressed by KCs. Therapeutic potential of RIPK1 kinase activity remains to be demonstrated in fulminant viral hepatitis.

In article 2, we investigated the role of RIPK1 in acute models of hepatitis induced by APAP and CCl4. Previous reports suggest that inhibition of RIPK1 kinase activity resulted in partial improvement in liver injury (134, 260-264, 280). However, these findings should be used with caution as Nec-1 is usually dissolved in DMSO, which can interfere in APAP metabolism and Nec-1 can have off–target effects (281-284). Nec-1s is a more specific inhibitor of RIPK1 kinase activity and is demonstrated to be efficient in minimizing APAP induced hepatocytes damage *in vitro* and *in vivo* (264). However, absence of DMSO control in this study remains the limitation (264). Future studies with vehicle control will be required to better understand the role of RIPK1 kinase activity in APAP induced hepatotoxicity. In accordance with previous data published by Schneider *et al.* (135), we demonstrated that *Ripk1* depletion specifically in liver parenchymal cells does not influence APAP induced hepatotoxicity (135). In contrast, knockdown of *Ripk1* through antisense oligonucleotide treatment efficiently reduced liver damage *in vivo*, demonstrating the protective role of RIPK1 protein in APAP induced liver damage (134). In our current study, we demonstrated that additional inhibition of RIPK1 kinase activity by pre-treatment of Nec-1s in *Ripk1*^{LPC-KO} mice does not protect from APAP-induced liver injury. This demonstrates that RIPK1 kinase activity in cells other than liver parenchymal cells is dispensable. Difference in susceptibility of genetical and pharmacological depletion of *Ripk1* to APAP-induced hepatotoxicity can be due to the differences between the methods

employed to deplete *Ripk1*, as genetically depleted mice never expressed RIPK1, while ASO-depleted RIPK1 transiently and partially. Furthermore, in agreement with previous studies, we confirmed that the type of cell death in APAP-induced hepatotoxicity is independent of caspase activation (285) and RIPK1-deficiency in liver parenchymal cells does not switch to apoptotic cell death as in ConA induced liver damage (167). In addition, mice deficient for RIPK3 or MLKL are not protected from APAP-induced liver damage, suggesting the absence of necroptosis (134). Collectively, these indicate that RIPK1 regulates necrotic cell death independently of apoptosis and necroptosis, and would probably be linked to the kinase activity of RIPK1 in liver parenchymal cells. To further address the role of RIPK1 kinase activity in APAP-induced hepatotoxicity, studies with administration of APAP in *Ripk1*[KD]mice should be conducted.

In parallel, we demonstrated that RIPK1–depletion, specifically in liver parenchymal cells, sensitizes *Ripk1*[LPC-KO] mice to CCl4-induced hepatocyte apoptosis. Increased hepatic expression of the liver transcript of the death ligand TNF-α, and elevated plasma concentrations of TNF-α are consistent features of CCl4-induced hepatotoxicity (119, 120, 286). Although TNF-α is involved in various liver pathologies (287), its role remains controversial in CCl4-induced hepatotoxicity (116). Mice deficient for TNFR1 or TNFR2 have similar liver damage in acute CCl4-induced hepatotoxicity (288, 289), suggesting that TNF signalling pathway is not decisive in CCl4–induced hepatitis. In contrast, other studies demonstrated that depletion of TNFR1 or neutralization of TNF-α could play protective role (117). However, in our experimental settings, the increased sensitivity of *Ripk1*[LPC-KO] to CCl4-induced hepatotoxicity is TNF-α independent as pre-treatment with ETA never improves liver injury. Previous reports suggest that varying dose of ETA can have protective or damaging effect (118). In contrast, we demonstrate that even varying doses of ETA do not change the susceptibility of mice to CCl4-induced liver toxicity. These conflicting reports on the role of TNF-α or its cognate receptors

could be due to differences in dose and route of administration of CCl4, in time of slaughtering post-administration of CCl4, or in strain or species of rodents. Additionally, commensal microbiota could affect the susceptibility of mice to CCl4-induced liver damage in chronic settings (290) and have also been describe to be involved in acute liver injury (291).

FASL is also involved in CCl4-induced hepatotoxicity, as mice deficient for FASL or neutralization of FASL resulted in improved liver injury (119). On the other hand, RIPK1 plays protective role in JO$_2$ (anti-FAS antibody) induced liver damage (166). This could explain the increased sensitivity of Ripk1^{LPC-KO} to CCl4-induced hepatotoxicity. However, future studies using of anti-FASL will be required to confirm this hypothesis.

In 3rd article, we determined the role of RIPK1 in NASH. Cell death carried out through death ligands plays pivotal role in the progression of NASH (32, 292-294). However, the therapeutic potential of death ligand inhibition has variable results on NASH progression (294, 295). Moreover, previous studies demonstrated that inhibition of RIPK1 kinase activity by Nec-1 results in amelioration of fatty acid toxicity in PMH and in NASH induced by methionine choline deficient diet (MCD) (48, 296). The limitation of this pharmacological approach is that Nec-1 can have additional off target effects and displays a short half-life (282). The role of RIPK1 kinase activity using specific inhibitors of RIPK1 kinase activity or genetic depletion of RIPK1 kinase activity remains to be explored in NASH. To elucidate the role of RIPK1 in NASH, we used high fat high cholesterol diet (HFHCD) to induce NASH in mice. We and others have already demonstrated that the HFHCD reproduces human histological lesions of NASH in liver, including hepatocyte ballooning and injury, inflammation, hepatic insulin resistance and extensive liver fibrosis (188, 297). It is important to note that this HFHCD does not induce obesity, which could be due to characteristic composition or decreased palatability.

Here we demonstrated that deletion of RIPK1, specifically in liver parenchymal cells, has a tendency to increase liver fibrosis, as compared to their wild-type Ripk1$^{fl/fl}$ littermates in HFHCD-induced NASH.

Recruitment of inflammatory cells in liver, is the key characteristic in the patients suffering from NASH (298). Here, we demonstrated that in early phase of HFHCD-induced NASH in mice, there is huge infiltration of inflammatory cells including Kupffer cells, neutrophils, lymphocytes, Regulatory T cells, NK cells and NKT cells. While during the late phase of NASH, the presence of kupffer cells and neutrophils remains elevated in liver, NKT cells were almost absent, in agreement with our previous study and others (297, 299-301). Our results give a notion that there is huge infiltration of inflammatory cells during the early phase of NASH, which shifts to low grade inflammation in the later phase of NASH. The depletion of NKT cells may be due to cell death induced by lipids. Indeed, the CD1d molecule, expressed at the surface of NKT, interacts with lipid antigens, and elevated quantities of fatty acids are known to promote apoptosis of NKT cells (301). Furthermore, the association between IL-12 (an NKT cell apoptosis inducer) and NKT apoptosis was demonstrated in diet-induced NASH in mice (300). Previous reports underline that NKT cells in diet induced NASH could vary according to differences in dietary models or to stage of NASH (301-304). As for our data, they showed that Ripk1 deficiency does not modulate the changes in immune populations during the course of NASH.

Chemokines and cytokines are important player in the development of NASH. In accordance with previous studies, we demonstrate that IL-6, TNFα and CCL2 were increased at transcript as well as at protein levels in liver (298, 300, 305). *In vitro*, Ripk1 deficiency impaired inflammatory cytokine production in LPS/poly I:C stimulated fibroblasts and monocytes (57) but here we observed that RIPK1 in liver parenchymal cells is dispensable for the production of inflammatory cytokines like TNFα, IL-6 and CCL2, as reflected by their respective liver

transcript levels and their plasma concentrations. Additionally, RIPK1-deficiency does not change the pattern of recruitment in the liver of inflammatory cells during the progression of HFHCD-induced NASH in mice.

Fibrosis is another key characteristic in pathogenesis of NASH. The histological determination of fibrosis remains a gold standard for the qualitative and quantitative diagnosis of liver fibrosis (306). Here, we demonstrated that RIPK1 deficiency resulted in increased liver fibrosis. This may be due to increased expression of TIMPs, inhibitors of metalloproteinases in charge of ECM degradation. Interestingly, liver transcripts of both MMPs and TIMPs were upregulated, which is in accordance with previous studies (307). Here, we show that RIPK1-deficiency resulted in increased expression of Nox-2 (a marker of oxidative stress), which is suggested to be involved in liver fibrosis (308). This could further be due to increased sensitivity of RIPK1 deficient mice to LPS as LPS absorption was increased from leaky gut in NASH and TLR4 knockout mice showed decreased fibrosis in dietary model of NASH (259, 309). Future studies elucidating the role of RIPK1 in oxidative stress in NASH, will pave the way to unveil the mechanism involved.

In conclusion, our study demonstrated that depletion of RIPK1 specifically in liver parenchymal cells has a tendency to increase liver fibrosis in HFD-induced NASH. Interestingly, the role of RIPK1 kinase activity in progression of NASH need to be addressed by using RIPK1 kinase dead or by pharmacological inhibition.

Liver fibrosis remains the strongest predictor of mortality in NASH patients (310, 311). Effect of dietary intervention on advanced fibrosis in diet-induced NASH is not well characterized. In article 4, we showed that in early non-fibrotic -NASH induced by 5 weeks of HFHCD, switching to standard chow diet for 4 weeks significantly ameliorated histological parameters

of NASH, and similarly, in advanced fibrotic NASH, dietary intervention improved liver steatosis, inflammation and oxidative stress. These data were in accordance with those obtained in other models of NASH (methionine choline deficient diet, choline deficient L-amino acid defined [CDAA] diet, and Western diet) (312-314). Additionally, we also demonstrated that a return to a standard diet resulted in cessation of liver damage, as evidenced by basal transaminase levels and decreased hepatomegaly. Besides, partial reversion of hepatic fibrosis was achieved in late-fibrotic NASH, when mice were kept under standard diet for 12 weeks. In comparison, liver fibrosis induced by chronic administration of CCl4 or alcohol in rodents resolved completely within 4-7 weeks after toxic removal (315-319). The partial resolution of fibrosis in our model coincided with a drop of liver transcripts of TIMPs and MMPs, without however returning to baseline levels. The importance of TIMPs regulation seems relevant since anti-TIMP1 antibodies improved CCl4-induced liver fibrosis in rodents (320). In contrast, another independent study showed persistent liver fibrosis in CDAA-induced NASH after a dietary intervention of 7 weeks (312). This discrepancy could be explained by species variation, stage of liver fibrosis, difference in diet composition and duration of dietary intervention. Further studies in this last model, with longer dietary intervention, will be required to resolve this discrepancy.

Taken together, our results demonstrate that dietary intervention improves hepatomegaly, liver damage, oxidative stress and inflammation in both fibrotic and non-fibrotic NASH. However, the process of liver fibrosis regression obtained after dietary intervention appeared slow. Therefore, therapeutic potential of dietary intervention in combination with anti-fibrotic agents needs to be explored.

Conclusion

In conclusion, my thesis work investigated the role of liver parenchymal cell specific RIPK1 in fulminant viral hepatitis, APAP-induced hepatotoxicity, CCl4-induced liver damage and in NASH. RIPK1 plays different role depending on the etiological agent. Thus, RIPK1 protects from fulminant viral hepatitis in a TNF-α dependent manner while its protective effect in CCl4--induced hepatotoxicity was TNF-α independent. In contrast, it is dispensable for APAP induced hepatotoxicity. Furthermore, mechanism of the RIPK1 protective properties in NASH-induced fibrosis remains unknown. As, inhibition of RIPK1 kinase activity can protect from both apoptosis and necroptosis and could be of added advantage in targeting cell death. Future studies will be required to elucidate the potential of RIPK1 kinase activity inhibition in various animal models and could be potential therapeutic agents in human liver pathologies.

<center>**Perspectives**</center>

Perspective 1. Evidence and role of necroptosis in various animal models of hepatitis.

Cell death is a key player during progression of liver diseases (32, 61). Previous studies suggested the presence of necroptosis in various liver pathologies based on pharmacological or genetic inhibition of RIPK1 and RIPK3. Today, it is known that RIPK1 and RIPK3 can have necroptosis-independent functions and non-canonical necroptosis can occur without involvement of RIPK1 or RIPK3 (50). However, determination of *in vivo* necroptosis remains challenging. Today, the detection of the phosphorylated form of MLKL by Western Blot is a useful tool to study the occurrence of necroptosis. Nonetheless, involvement of necroptosis in hepatocytes during chronic liver diseases such as NASH and ALD remains highly controversial. The establishment of a MLKL^{LPC-KO} mouse line will be a potential relevant tool to investigate this specific aspect. Unfortunately, no pharmacological inhibitor of murine MLKL is available. Development of such new MLKL inhibitors will help to further improve our understanding. Inhibition of cell death can only be useful to address the progression of liver disease. However, in cell death resistant cancerous cell, induction of cell demise can be of therapeutic value. In line with this one study recently demonstrated that induction of necroptosis through intratumorally MLKL mRNA delivery resulted in regression of melanoma and increased tumour immunity (321). Induction of necroptosis can be exploited in HCC with the advancement of targeted drug delivery approach.

Perspective 2. Role of RIPK1 kinase activity in APAP induced hepatotoxicity.

Various studies demonstrated some protection from hepatic injury due to overdose of APAP by inhibiting RIPK1 kinase activity through pre-treatment with Nec-1 or Nec-1s in mice models (50). It is important to note that Nec-1 can display off–target effects. While Nec-1s is more specific, only one study used it and described it as an efficient protective agent against APAP-induced hepatotoxicity. However, conclusions can be questioned since the DMSO vehicle control is lacking, although this solvent has already been described as a molecule capable to limit hepatitis resulting from APAP overdose. Therefore, no conclusion can be definitively established with this approach until these experiments are reproduced with Nec-1S by including the control with DMSO alone, which will serve as the reference for the basic level of APAP-induced hepatotoxicity. Another feasible approach will be the exploitation of RIPK1 kinase dead knock-in mice (*Ripk1*[k45A]). In parallel, candidate molecules should be tested on primary cultures of mouse and human hepatocytes. It is only after carrying out all these pre-clinical studies of efficacy and safety that these inhibitors can be evaluated for therapeutic applications in humans.

Perspective 3. Role of liver parenchymal specific RIPK1 in Alcoholic liver disease.

According to WHO report, alcohol is attributed to 13.5% of all deaths in age group of 20-39 worldwide. Depending on dose, alcohol consumption is a risk factor for about 200 diseases. Among these, we distinguish alcoholic liver disease (ALD) that can progress into cirrhosis and HCC. Hepatocyte death demonstrated to be key regulator in ALD and depletion of RIPK3 has been shown to improve ALD-induced liver injury (44). However, pre-treatment of Nec-1 did not improve liver injury in this model. The author proposed that it could be due to short half-life of Nec-1. However, a recent study demonstrated that inhibition of RIPK1 kinase activity by single injection of Nec-1s before binge did not improve liver injury but improved the

inflammation in the NIAAA model of alcoholic liver injury (chronic and binge ethanol feeding) (144). However, in this case Nec-1s was just administered before binge ethanol feeding. It will be interesting to exploit the role of inhibition of RIPK1 kinase activity during the course of chronic and binge ethanol feeding. To further assess the role of RIPK1 kinase activity, genetic model can be employed (*Ripk1*^k45A). Additionally, our data demonstrate that the function of RIPK1 is context dependent, opening avenues to explore the role of RIPK1 in ALD.

Perspective 4. Inhibition of regulated cell death (apoptosis and necroptosis) in chronic liver diseases.

Hepatocyte death plays central role in the progression of chronic liver diseases. However, targeting cell death as a therapeutic approach for chronic liver diseases should be only considered according to the advancement status of the disease. Thus, it will be relevant to inhibit cell death during the hepatitis phase, but to promote cell death for resistant cancerous cells. Apoptosis is the most studied form of cell death in liver diseases. Currently, there is no FDA approved treatment for NASH. Emericasan, a pan-caspase inhibitor, is in phase II clinical trial for the NASH treatment. However, HFD feeding resulted in increased RIPK3 expression in both human and mice (48). Moreover, liver parenchymal specific deletion of Casp-8 resulted in increased NASH-induced liver damage accompanied by increased pMLKL positive area in liver sections. These data suggested a potential switch to necroptosis (61). Despite this, the involvement of necroptosis in NASH is not fully proven. Thus, it would be all the more interesting to reproduce these NASH HFD-induced experiments on mice with double knockout of Casp-8 and MLKL. Long term follow-up of these double knockout mouse line will be required to assess that resistance to cell death does not promote the emergence of cancer cells and thus HCC. RIPK1 kinase activity, being involved in both apoptosis and necroptosis, can also be a relevant target to mitigate the progression of chronic liver diseases. There is only limited information on the role of RIPK1 kinase activity in NASH and ALD. Additional studies

are therefore needed to evaluate the relevance of this therapeutic target. The RIPK1 kinase activity inhibitor (GSK2982772) is already in phase II clinical trial for rheumatoid arthritis, psoriasis and inflammatory bowel disease. Its potential as a therapeutic agent also need to be addressed in chronic liver diseases (273). Similarly, therapeutic potential of combined inhibition of apoptosis and necroptosis need to be assessed in ALD.

In conclusion, targeting apoptosis and necroptosis may provide an important therapeutic approach during the progression of chronic liver diseases. Thus, study of therapeutic potential of cell death in liver diseases is in early stage and require further research.

V. RESUME

La mort cellulaire joue un rôle central dans le développement et la progression des maladies du foie (32, 61). En condition physiologique, un faible nombre de cellules meurent sans induire de réaction inflammatoire. Cependant, lors d'une agression du foie d'origine virale, bactérienne, toxique ou auto-immune, des hépatocytes meurent, induisant une inflammation et une prolifération compensatrice. Lorsque ce phénomène est chronique, l'inflammation, induite par les motifs moléculaires associés aux dégâts ou par les corps apoptotiques, peut conduire à une fibrose, voire à un carcinome hépatocellulaire (32, 61), le $3^{ième}$ cancer le plus mortel (269). Quel que soit l'agent étiologique, l'expression et la sécrétion de ligands de mort (TNFα, FASL et TRAIL) par des cellules inflammatoires sont des éléments clés de la progression des maladies du foie. Sachant que la mort cellulaire peut subvenir de différentes manières, telles que l'apoptose ou la nécroptose, leurs inhibitions peuvent revêtir un intérêt thérapeutique (32, 61, 270).

Le protéine kinase cytosolique RIPK1 influe sur le devenir de la cellule, en favorisant soit la survie, soit la mort cellulaire par apoptose dépendante de caspases ou par nécroptose dépendante de RIPK3 et MLKL. Cette protéine pourrait donc être une cible potentielle dans la régulation de la mort cellulaire. Cette kinase joue un rôle clé dans la réponse à divers stimuli tels que, par exemple, les ligands de mort (TNFα, L de FAS et TRAIL), la surcharge calcique, le stress du réticulum endoplasmique, les dommages à l'ADN, l'interférons, les motifs moléculaires associés aux pathogènes (LPS bactérien et ARNdb viral) (153, 198-205). Alors que l'activité kinase de RIPK1 favorise la mort cellulaire, ses propriétés d'échafaudage contribuent à la survie. Jusqu'à présent, il a été démontré que RIPK1 joue un rôle protecteur au cours d'hépatites induites par la lectine ConA (modèle d'hépatite auto-immune), par des ligands de mort (TNFα, anti-FAS et la combinaison TRAIL / IFN gamma) ou par le LPS.

En ce qui concerne le rôle de RIPK1 dans l'hépatite virale fulminante, rien n'était connu jusque-là. Ainsi, dans l'article n°1, notre objectif était de l'explorer en comparant les symptômes développés chez des souris déficientes pour RIPK1 seulement au sein des cellules du parenchyme hépatique (*Ripk1*$^{LPC-KO}$) et des souris témoins au phénotype sauvage (*Ripk1*$^{fl/fl}$), durant une hépatite virale fulminante induite par le virus MHV3. L'inoculation a induit une augmentation progressive des marqueurs de l'infection et de l'inflammation (TNFα et CCL2), chez les 2 génotypes de souris. Bien que tous les animaux soient morts 4 jours après l'infection,

les souris *Ripk1*[LPC-KO] sont apparues plus sensibles, comme en témoignent les niveaux plus élevés de transaminases plasmatiques et d'apoptose dans le tissu hépatique à des temps plus précoces. Collectivement, ces données ont démontré que l'absence de RIPK1 sensibilise les souris à l'hépatite induite par le MHV3. Aujourd'hui, il reste à explorer le rôle de l'activité kinase de RIPK1 au cours de cette affection. Pour comprendre les mécanismes impliqués dans la mort cellulaire accrue observée chez les souris *Ripk1*[LPC-KO] infectées par le MHV3, nous avons utilisé un modèle d'injection d'ARN synthétique double brin, le poly I:C, qui imite la présence de formes réplicatives de génomes viraux. Son administration seule a entraîné des dommages hépatiques seulement chez les souris *Ripk1*[LPC-KO]. Un prétraitement avec de l'ETA (un récepteur leurre du TNF-α) a permis de prévenir les lésions hépatiques induites par le poly I:C, démontrant ainsi le rôle potentiel du TNF-α. En outre, une protection a pu être également obtenue par déplétion préalable des macrophages. Par conséquent, le TNF-α, responsable des dommages hépatiques, étaient probablement exprimé par les cellules de kupffer (KCs), suite à la stimulation du TLR3. Pris ensembles, ces données illustrent le rôle protecteur joué par RIPK1 contre le TNFα produit par les KCs via la stimulation du récepteur TLR3 au cours de l'hépatite.

Dans l'article n°2, nous avons étudié le rôle de RIPK1 dans des modèles d'hépatites aiguës induites par le paracétamol ou le tétrachlorométhane (CCl4). Des rapports antérieurs ont suggéré que l'inhibition de l'activité kinase de RIPK1 pouvait partiellement améliorer les lésions hépatiques induites par le paracétamol (134, 260-264, 280). Cependant, l'utilisation d'un inhibiteur non spécifique ou l'absence de contrôles adaptés demeurait la limite de ces études. Nos travaux démontrent que la déplétion de Ripk1, spécifiquement dans les cellules du parenchyme hépatique, n'influence pas l'hépatotoxicité induite par le paracétamol. De plus, l'inhibition supplémentaire de l'activité kinase de RIPK1 par un prétraitement avec du Nec-1s n'a pas protégée les souris *Ripk1*[LPC-KO] des lésions hépatiques induites par le paracétamol. Cela démontre que l'activité kinase de RIPK1, dans des cellules autres que les cellules parenchymateuses du foie, ne participe pas aux dommages hépatiques induits par le paracétamol. De plus, en accord avec des études antérieures, nous avons confirmé que le type de mort cellulaire dans l'hépatotoxicité induite par le paracétamol est indépendant de l'activation des caspases (285) et que le déficit en RIPK1 dans les cellules parenchymateuses du foie ne modifie par le type de mort en favorisant l'apoptose, comme dans l'hépatite auto-immune induite par la lectine ConA (167). Par ailleurs, les souris déficientes en RIPK3 ou MLKL ne sont pas protégées contre les lésions hépatiques induites par le paracétamol, ce qui suggère l'absence de nécroptose (134). Collectivement, toutes ces données indiquent que RIPK1 régule

une mort cellulaire autre que l'apoptose ou la nécroptose, et serait probablement liée à l'activité kinase de RIPK1 dans les cellules du parenchyme du foie.

En parallèle, nous avons démontré que la déplétion de RIPK1, en particulier dans les cellules du parenchyme hépatique, sensibilise les hépatocytes des souris *Ripk1*^{LPC-KO} à l'apoptose induite par le CCl4. Le rôle du TNFα est très controversé dans ce modèle (116). Cependant, dans nos expériences, la sensibilité accrue des souris *Ripk1*^{LPC-KO} à l'hépatotoxicité induite par le CCl4 est indépendante du TNFα, car un prétraitement à l'ETA n'a jamais amélioré les lésions hépatiques. Contrairement à une précédente étude(118), nous avons constaté que même des doses croissantes d'ETA ne modifiaient pas la sensibilité des souris *Ripk1*^{LPC-KO} à la toxicité hépatique induite par le CCl4. Ces données contradictoires sur le rôle du TNFα ou de ses récepteurs apparentés pourraient être dues à des différences de dose et de voie d'administration du CC14, au moment du sacrifice post-administration du CC14, ou à une souche ou une espèce de rongeur. En outre, le microbiote commensal peut affecter la sensibilité des souris aux dommages hépatiques induits par le CC14 dans un contexte chronique (290) ou aiguës (291). FASL est également impliqué dans l'hépatotoxicité induite par le CC14, car la déficience en FASL, ou la neutralisation de FASL, limite la survenue des lésions hépatiques (119). Or, RIPK1 joue un rôle protecteur dans les dommages hépatiques induits par JO2 (anticorps anti-FAS) (166). Cela pourrait expliquer l'augmentation de la sensibilité des souris *Ripk1*^{LPC-KO} à l'hépatotoxicité induite par CCl4. Des investigations supplémentaires seront nécessaires pour tester cette hypothèse.

Dans le 3ième article, nous avons exploré le rôle de RIPK1 au cours de la stéato-hépatite non alcoolique (NASH). La mort cellulaire induite par des ligands de mort joue un rôle central dans la progression de la NASH (32, 292-294). Cependant, l'inhibition de ligands de mort présente des résultats mitigés sur la progression de la NASH (294, 295). Pour élucider le rôle de RIPK1 au cours de la NASH murine, nous avons utilisé un régime riche en graisses et en cholestérol (HFHCD) pour reproduire les lésions histologiques de la NASH retrouvées dans le foie de patients, y compris l'augmentation du volume des hépatocytes (ballooning), les lésions, l'inflammation, la résistance hépatique à l'insuline et la fibrose hépatique (188, 297). Le recrutement de cellules inflammatoires dans le foie est la principale caractéristique chez les patients atteints de NASH (298). Ici, nous avons démontré que dans la phase précoce de la

NASH induite par HFHCD chez la souris, il y avait une infiltration très importante de cellules inflammatoires, comprenant des KC, des neutrophiles, des lymphocytes, des cellules T régulatrices, des cellules NK et NKT. Dans la NASH avancée, alors que la présence de KC et de neutrophiles demeurait élevée dans le foie, les cellules NKT étaient presque absentes, ce qui concorde avec des études antérieures dont les nôtres (297, 299-301). Nos résultats suggèrent qu'il existe une énorme infiltration de cellules inflammatoires au cours de la phase initiale de la NASH, laquelle passe ensuite à une inflammation de bas grade. Par ailleurs, nous avons constaté que le déficit en RIPK1 ne modulait jamais les changements observés au sein des populations immunes du foie au cours de la NASH. Cependant, le déficit en RIPK1 entraînait une fibrose hépatique accrue. Cela peut être dû à l'augmentation de l'expression des TIMPs, des inhibiteurs de métalloprotéinases (MMPs) normalement responsables de la dégradation de la matrice extracellulaire. De plus, l'expression de Nox-2 (un marqueur du stress oxydatif), qui est supposé être impliqué dans la fibrose hépatique (308), a augmenté chez les souris $Ripk1^{\text{LPC-KO}}$. L'aggravation de la fibrose observée chez les souris $Ripk1^{\text{LPC-KO}}$ pourrait également être liée à la sensibilité accrue de ces souris au LPS, puisque de plus grandes fuites intestinales se produisent au cours de la NASH, en raison d'une augmentation de la perméabilité de l'intestin, mais aussi parce que les souris TLR4 knock-out présentent une diminution de la fibrose dans un modèle de NASH induite par un régime alimentaire déséquilibré (259, 309).

La fibrose du foie est un facteur important de la prédiction de la mortalité chez les patients atteints d'une NASH (310, 311), mais l'effet de l'intervention diététique sur une fibrose avancée reste à évaluer. Dans l'article n°4, nous avons montré que dans une NASH précoce non fibreuse (induite par 5 semaines de HFHCD), un passage à un régime alimentaire standard pendant 4 semaines améliore sensiblement les paramètres histologiques de la NASH. De même, lors d'une NASH fibreuse avancée, l'intervention diététique améliore la stéatose hépatique, l'inflammation et le niveau de stress oxydatif. Ces données sont conformes à celles obtenues dans d'autres modèles de NASH (alimentation déficiente en choline et méthionine [MCD], régime défini en acides aminés et déficient en choline [AADC] et régime occidental) (312-314). En outre, nous avons également démontré qu'un retour à un régime normal abouti à un arrêt de l'atteinte hépatique, comme en témoignent les niveaux de base en transaminases sériques et la diminution de l'hépatomégalie. Enfin, une réversion partielle de la fibrose hépatique a été obtenue chez les animaux initialement atteints d'une fibrose avancée, lorsque les souris étaient maintenues sous un régime normal pendant 12 semaines. La résolution partielle de la fibrose

dans notre modèle a coïncidé avec une baisse des transcrits hépatiques des TIMPs et des MMPs, sans toutefois revenir à des niveaux normaux.

Conclusion

Mon travail de thèse a abordé le rôle de RIPK1 dans les cellules du parenchyme hépatique au cours de différentes hépatites : virale fulminante, induite par le paracétamol ou le CCl4 ou lors d'une NASH. Cette protéine joue un rôle différent selon l'agent étiologique. Ainsi, RIPK1 protège les hépatocytes du TNFα lors d'une hépatite virale fulminante, alors que son effet protecteur au cours de l'hépatotoxicité induite par le CCl4 était indépendant du TNFα. En revanche, sa présence n'influence pas l'hépatotoxicité induite par le paracétamol. En outre, RIPK1 limite la fibrose induite lors de la NASH, par des mécanismes qui restent à déterminer. Puisque l'inhibition de l'activité kinase de RIPK1 peut protéger à la fois de l'apoptose et de la nécroptose, il sera intéressant dans de futures études d'étudier l'effet de cette inhibition dans divers modèles d'hépatites animales, afin d'évaluer son potentiel thérapeutique au cours des pathologies humaines du foie.

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VII. ANNEXURE

RIPK1 protects from TNF-α-mediated liver damage during hepatitis

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Cell death of hepatocytes is a prominent characteristic in the pathogenesis of liver disease, while hepatolysis is a starting point of inflammation in hepatitis and loss of hepatic function. However, the precise molecular mechanisms of hepatocyte cell death, the role of the cytokines of hepatic microenvironment and the involvement of intracellular kinases, remain unclear. Tumor necrosis factor alpha (TNF-α) is a key cytokine involved in cell death or survival pathways and the role of RIPK1 has been associated to the TNF-α-dependent signaling pathway. We took advantage of two different deficient mouse lines, the RIPK1 kinase dead knock-in mice (Ripk1[K45A]) and the conditional knockout mice lacking RIPK1 only in liver parenchymal cells (Ripk1[LPC-KO]), to characterize the role of RIPK1 and TNF-α in hepatitis induced by concanavalin A (ConA). Our results show that RIPK1 is dispensable for liver homeostasis under steady-state conditions but in contrast, RIPK1 kinase activity contributes to caspase-independent cell death induction following ConA injection and RIPK1 also serves as a scaffold, protecting hepatocytes from massive apoptotic cell death in this model. In the Ripk1[LPC-KO] mice challenged with ConA, TNF-α triggers apoptosis, responsible for the observed severe hepatitis. Mechanism potentially involves both TNF-independent canonical NF-κB activation, as well as TNF-dependent, but canonical NF-κB-independent mechanisms. In conclusion, our results suggest that RIPK1 kinase activity is a pertinent therapeutic target to protect liver against excessive cell death in liver diseases.
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Contemporary liver diseases result from chronic conditions, such as chronic viral hepatitis, nonalcoholic fatty liver hepatitis (NASH) and alcoholic liver hepatitis.[1] The global pandemic of chronic viral hepatitis (hepatitis B and C virus infections) affects a significant proportion of the world population, currently estimated at around 500 million people.[2] In parallel, the prevalence of NASH rises especially in Western countries because of lifestyle evolution. Untreated, chronic hepatitis predispose to the development of cirrhosis and hepatocellular carcinoma (HCC).[3] The progression of these diseases is triggered by hepatocyte death,[3,4] and the cell death processes are starting to emerge. Immune cells, including liver resident macrophages (Kupffer cells) or infiltrating natural killer (NK) cells and T cells (natural killer T (NKT) and T lymphocytes), produce molecules that induce hepatic parenchyma damage. Immune cells secrete or expressed at their surface tumor necrosis factor alpha (TNF-α), FAS ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL). These ligands induce cell death by engaging their respective receptors (TNFR, Fas and death receptors 4 and 5 (DR4, DR5)) present at the surface of hepatocytes.[5] Receptor interacting protein kinase 1 (RIPK1) is a key protein regulating signaling downstream of these DRs, and is best characterized for its roles downstream of TNFR1.[6] Clinical studies have underlined the crucial role of TNF-α in several liver diseases. Indeed, serum TNF-α levels increase in patients with fulminant hepatic failure[7] and is correlated with poor prognostic.[8] TNF-α is a master pro-inflammatory cytokine that binds TNFR1 and TNFR2, but most of its biological activities have been associated with TNFR1 signaling. Downstream of TNFR1, RIPK1 functions as a signaling node driving cell survival as well as caspase-8-dependent apoptosis or RIPK3/mixed lineage kinase domain-like pseudokinase (MLKL)-dependent necroptosis.[9] These opposed cellular fates are regulated by two different faces of RIPK1, it functions as a scaffold to promote cell survival, in part via NF-κB activation, and as a kinase to induce cell death.[6] The perinatal lethality of RIPK1-deficient mice has long hampered the *in vivo* study of the dual faces of RIPK1.[10] Recently, the publication of viable and healthy RIPK1

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Abbreviations: AST, aspartate amino-transferase; ALT, alanine amino-transferase; ConA, concanavalin A; DR, death receptor; ETA, etanercept; HCC, hepatocellular carcinoma; H&E, hematoxylin and eosin coloration; i.v, intravenous; i.p, intraperitoneal; NASH, nonalcoholic fatty liver hepatitis; Nec-1, necrostatin-1; P.I, post-injection; RIPK, receptor interacting protein kinase; Q-VD-OPh, quinoline-Val-Asp-CH$_2$-Ph; TNF, tumor necrosis factor; TRAF2, TNF receptor-associated factor 2; TRAIL, TNF-related apoptosis-inducing ligand
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Research Article

Endogenous IL-33 Deficiency Exacerbates Liver Injury and Increases Hepatic Influx of Neutrophils in Acute Murine Viral Hepatitis

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The alarmin IL-33 has been described to be upregulated in human and murine viral hepatitis. However, the role of endogenous IL-33 in viral hepatitis remains obscure. We aimed to decipher its function by infecting IL-33-deficient mice (IL-33 KO) and their wild-type (WT) littermates with pathogenic mouse hepatitis virus (L2-MHV3). The IL-33 KO mice were more sensitive to L2-MHV3 infection exhibiting higher levels of AST/ALT, higher tissue damage, significant weight loss, and earlier death. An increased depletion of B and T lymphocytes, NKT cells, dendritic cells, and macrophages was observed 48 h postinfection (PI) in IL-33 KO mice than that in WT mice. In contrast, a massive influx of neutrophils was observed in IL-33 KO mice at 48 h PI. A transcriptomic study of inflammatory and cell-signaling genes revealed the overexpression of IL-6, TNFα, and several chemokines involved in recruitment/activation of neutrophils (CXCL2, CXCL5, CCL2, and CCL6) at 72 h PI in IL-33 KO mice. However, the IFNγ was strongly induced in WT mice with less profound expression in IL-33 KO mice demonstrating that endogenous IL-33 regulated IFNγ expression during L2-MHV3 hepatitis. In conclusion, we demonstrated that endogenous IL-33 had multifaceted immunoregulatory effect during viral hepatitis via induction of IFNγ, survival effect on immune cells, and infiltration of neutrophils in the liver.

1. Introduction

The cytokine IL-33 is the 11th member of the IL-1 family and is designated as IL-1F11. It is now described as a DAMP (damage-associated molecular patterns) or "alarmin" molecule that is normally restrained to the nuclear compartment where it could act as a nuclear factor-regulating gene expression [1] but is released in case of pathogen aggression or

RIPK1 protects hepatocytes from death in Fas-induced hepatitis

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Hepatocyte death is a central event during liver disease progression, in which immune cells play key roles by activating members of the Tumor Necrosis Factor Receptor Superfamily (TNFRSF), including TNFR1 (TNFRSF1A), Fas (TNFRSF6) and TRAIL-R2 (TNFRSF10B). Receptor Interacting Protein Kinase 1 (RIPK1) emerged as a signaling node downstream of these receptors. In the case of TNFR1, RIPK1 has been demonstrated to paradoxically serve as a scaffold to promote the survival of hepatocytes and as a kinase to kill them. To evaluate whether RIPK1 also protects hepatocytes from death in response to FasL or TRAIL, we took advantage of liver parenchymal cell-specific *Ripk1* knockout mice (*Ripk1*[LPC-KO]). We found that *Ripk1*[LPC-KO] mice, as well as primary hepatocytes derived from them, were more susceptible to Fas-mediated apoptosis than their respective WT counterparts. Fas-induced hepatocyte death was independent of TNF-α signaling. Interestingly, while TRAIL administration did not induce hepatitis in *Ripk1*[LPC-KO] mice or in their WT counterparts, its combination with IFN-γ only induced TNF-α dependent apoptosis in the *Ripk1*[LPC-KO] mice. Together, our data demonstrate the protective role of RIPK1 downstream of Fas and highlight the general protective function of RIPK1 in hepatocytes exposed to inflammatory conditions, where TNF-α, FasL and/or TRAIL are present.

The intravenous injection of the plant lectin Concanavalin A (ConA) is a widely used experimental model for acute immune-mediated hepatitis in mice. The ConA carbohydrate-binding protein is known to induce both activation and recruitment of immune cells (such as natural killer T (NKT) –cells) to the liver where they drive inflammation and death of hepatocytes. Accordingly, depletion of NKT-lymphocytes or of the invariant-NKT subpopulation avoids liver injury normally induced by ConA[1–3]. During liver diseases, members of the Tumor Necrosis Factor Superfamily (TNFSF), such as TNF-α, FasL (or Apo1/CD95) and TNF-Related Apoptosis Inducing Ligand (TRAIL) are expressed and released by immune cells, and can directly or indirectly trigger hepatocyte death[4, 5]. TNF-α and IFN-γ indirectly participate in liver injury by promoting favorable inflammatory conditions dependent on non-parenchymal cells. Indeed, specific deletion of TNFR1 in myeloid-derived cells, but not in liver parenchymal cells, protects from ConA-induced hepatitis[6], while IFN-γ has been shown to promote NKT-activation[7]. In contrast, TRAIL and FasL, the main cytokines expressed by the hepatic NK- and NKT-cells in the ConA model, are most likely the cytokines directly responsible for the death of hepatocytes since their inhibition or deletion prevent ConA-induced liver damage[2, 8–12]. In line with this idea, the mouse liver is highly sensitive to a single injection of FasL[13] or of a Fas agonist[14]. It induces fulminant hepatitis by triggering acute hepatocyte and liver sinusoidal endothelial cell (LSEC) death in a dose dependent manner, leading to liver hemorrhages and death of the mice[13–15]. A single injection of TRAIL does not induce hepatolysis, but inhibition of the TRAIL pathway protects mice against ConA-induced liver damage[8, 12], supporting a pro-death role played by TRAIL in the inflammatory context generated by ConA.

The Receptor Interacting Protein Kinase 1 (RIPK1) has been identified as part of the cellular responses induced by members of the TNF superfamily, including TNF-α[16], FasL[17] and TRAIL[18, 19]. Intense efforts have been made in the last decade to better understand the role of RIPK1 in TNF-α signaling, where it was reported to serve as a signaling node controlling the life/death cell-fate switch[20–22]. RIPK1 was shown to function as a scaffold

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RIPK1 protects hepatocytes from Kupffer cells-mediated TNF-induced apoptosis in mouse models of PAMP-induced hepatitis

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See Editorial, pages 1118–1119

Background & Aims: The severity of liver diseases is exacerbated by the death of hepatocytes, which can be induced by the sensing of pathogen associated molecular patterns (PAMPs) derived from the gut microbiota. The molecular mechanisms regulating these cell death pathways are poorly documented. In this study, we investigated the role of the receptor interacting protein kinase 1 (RIPK1), a protein known to regulate cell fate decisions, in the death of hepatocytes using two *in vivo* models of PAMP-induced hepatitis.

Methods: Hepatitis was induced in mice by independent injections of two different bacterial PAMPs: lipopolysaccharide (LPS) and unmethylated CpG oligodeoxynucleotide (CpG-DNA) motifs. The role of RIPK1 was evaluated by using mice specifically lacking RIPK1 in liver parenchymal cells (*Ripk1*[LPC-KO]). Administration of liposome-encapsulated clodronate served to investigate the role of Kupffer cells in the establishment of the disease. Etanercept, a tumor necrosis factor (TNF)-decoy receptor, was used to study the contribution of TNF-α during LPS-mediated liver injury.

Results: Whereas RIPK1 deficiency in liver parenchymal cells did not trigger basal hepatolysis, it greatly sensitized hepatocytes to apoptosis and liver damage following a single injection of LPS or CpG-DNA. Importantly, hepatocyte death was prevented by previous macrophage depletion or by TNF inhibition.

Conclusions: Our data highlight the pivotal function of RIPK1 in maintaining liver homeostasis in conditions of macrophage-induced TNF burst in response to PAMPs sensing.

Lay summary: Excessive death of hepatocytes is a characteristic of liver injury. A new programmed cell death pathway has been described involving upstream death ligands such as TNF and downstream kinases such as RIPK1. Here, we show that in the presence of LPS liver induced hepatic injury was due to secretion of TNF by liver macrophages, and that RIPK1 acts as a powerful protector of hepatocyte death. This newly identified pathway in the liver may be helpful in the management of patients to predict their risk of developing acute liver failure.

Introduction

The liver is constantly exposed to bacterial pathogen associated molecular patterns (PAMPs) such as unmethylated CpG-DNA motifs or lipopolysaccharides (LPS) originating from the microbiota of the gastrointestinal tract. In physiological conditions, the intestinal barrier prevents the translocation of large amounts of bacterial by-products to the liver [1]. The low quantities of bacterial debris reaching the liver are efficiently cleared by phagocytic hepatic cells, avoiding induction of inflammation and harmful response [2]. In chronic liver diseases of steatosis or alcoholic origin, this intestine barrier function is damaged [3,4], resulting in an abnormal elevation of PAMPs in the liver and impairing liver homeostasis [5]. An increase in PAMPs in the liver is one of the most common factors responsible for the outbreak of acute hepatitis on chronic liver failure background (ACLF for acute on chronic liver failure) [6]. According to the European Association for the Study of Liver Disease (EASL) and the Asian Pacific Association for the Study of the Liver (APASL), ACLF is an acute deterioration of a pre-existing chronic liver disease, and as a consequence, can provoke high short-term mortality. Thus today, ACLF is one of the most challenging fields in hepatology [7]. However, the molecular mechanisms responsible for the liver failure are not completely understood.

The gut has a strong anatomical link with the liver, which plays a key role in the bacterial clearance, and explains why ~80% of macrophages are present in the liver [5,8]. These liver

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and THP-1 cells co-cultured with HCV^{+} or HCV^{−} Huh7.5 cells. Furthermore, CD14^{+} monocytes isolated from chronically HCV-infected individuals and HS were transfected with miR155 mimics, miR155 inhibitors or negative control. TNF-α and JNK1, 2, 3 were detected by ELISA and RT-PCR. T-bet, IL-12 and Il-10 were tested by flow cytometry. SOCS1 were detected by WB and RT-PCR.

Results: We demonstrated that miR-155 was up-regulated in CD14^{+} M/M$_\Phi$ in HCV-infected patients compared to healthy subjects. Upon LPS, HCV core or NS5 stimulation, miR-155 was up-regulated in monocytic THP-1 cells. Up-regulation of miR-155 was also observed in THP-1 cells incubated with HCV^{+} Huh7.5 cells. Moreover, up-regulation of miR-155 in CD14^{+}M/M$_\Phi$ from HCV-infected patients induced TNF-α production and JNK signaling activation, leading to T-bet up-regulation, thereby, promoting inflammatory responses. Also, miR-155 up-regulation in CD14^{+} M/M$_\Phi$ of HCV-infected patients resulted in increased IL-12 and decreased IL-10 productions via inhibiting the suppressor of cytokine signaling 1 (SOCS1) signaling.
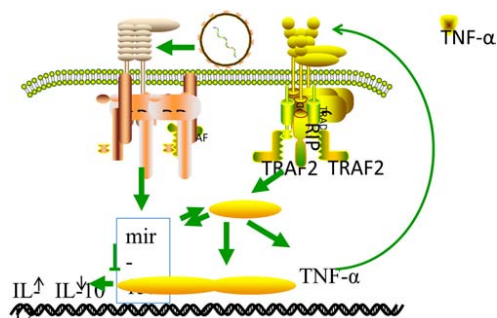

Figure: A model for miR155 regulation of monocyte functions in the setting of chronic HCV infection.

Conclusion: These results indicate that miR-155 up-regulation in M/M$_\Phi$ during HCV infection enhances the activation of TNF-α and JNK pathways to promote the expression of transcription factor T-bet and triggers pro-as well as anti-inflammatory mediators, such as IL-12, TNF-α, and IL-10, via the SOCS1 signaling. Our data reveal new information for elucidating the mechanisms of chronic HCV infection.

SAT-406
Depletion of RIPK1 in hepatocytes exacerbates PolyI:C and Murine Hepatitis Virus induced liver damage

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Background and Aims: The protein kinase RIPK1 plays a crucial role on the crossroad of stress-induced signaling pathways that affects cell's decision to live or die. Thus, RIPK1 is involved in the signal transduction pathway activated during the recognition of viral pathogen-associated molecular patterns (PAMPs) by the Toll-like receptor 3 (TLR3). The present study aimed to define the role of RIPK1 in hepatocytes when this pathway is stimulated with Poly I:C, a synthetic analog of double-stranded RNA or when a fulminant hepatitis is induced by Murine Hepatitis Virus type 3 (MHV3) infection.

Method: Mice deficient for RIPK1 specifically in liver parenchymal cells (RIPK1[LPC-KO]) and their wild-type littermates (RIPK1^{fl/fl}) have been challenged by Poly I:C. Etanercept, a TNF alpha receptor decoy, or clodronate encapsulated liposomes were injected prior administration of Poly I:C to explore the respective role of TNF alpha and macrophages in induced hepatitis. Primary cultures of mouse

hepatocytes isolated from RIPK1[LPC-KO] or RIPK1^{fl/fl} mice were also established to investigate the direct impact of Poly I:C on normal or deficient liver parenchymal cells. Besides, fulminant hepatitis induced by MHV3 were compared in RIPK1[LPC-KO] and RIPK1^{fl/fl} mice. Different clinico-pathological investigations (ALT, liver histology, qPCR…) were conducted to analyze challenged animals.

Results: Administration of Poly I:C led to increased serum ALT in RIPK1[LPC-KO] mice, reflecting liver damage through elevated apoptosis as illustrated by cleaved-caspase 3 labeling. Prior neutralization of TNF alpha protected hepatocytes from apoptosis in Poly I:C challenged RIPK1[LPC-KO] mice. Besides, depletion of macrophages also resulted in complete protection. In *in vitro* primary mouse hepatocytes, Poly I:C never induced direct cell death whatever the murine genotype, while it always stimulated an anti-viral response as characterized by the induction of PKR, Mx1 and OAS gene expression even in absence of RIPK1. Additionally, it has been discovered that RIPK1[LPC-KO] mice were more sensitive to MHV3 induced fulminant hepatitis.

Conclusion: Our data sustain the protective role played by RIPK1 in hepatocytes during viral induced fulminant hepatitis which is carried out through TNF alpha secreted from macrophages.

SAT-407
A novel chimpanzee adenoviral vectored HBV vaccine, encoding multiple HBV antigens with a shark invariant chain adjuvant, for use in HBV immunotherapy

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Background and Aims: Chronic hepatitis B virus (HBV) infects 257 million people globally. Current therapies suppress HBV but rebound occurs on cessation of therapy; novel therapeutic strategies are urgently required. To develop a potent therapeutic HBV vaccine that will induce T-cells to all major HBV antigens and generate anti-HBs antibodies, using Chimpanzee adenoviral vectors and shark class-II invariant (sIi) chain as a genetic adjuvant.

Method: We designed two HBV immunogens SIi-HBV-CP$_{mut}$S and HBV-CP$_{mut}$S; encoding precore (PreC), core, non-functional polymerase (P$_{mut}$), PreS1, PreS2 and surface antigens (Figure 1a). The large envelope protein of HBV (composed of PreS1, PreS2 and surface proteins) was generated separately using furin 2A (F2A) peptide cleavage protein. A 26 amino-acid sequence, derived from shark invariant chain (sIi), was inserted at the 5′ end of the SIi-HBV-CPmutS immunogen. The immunogens were encoded in chimpanzee-adenoviral vector (ChAdOx2) and tested in naïve mice given i.m. HBV-specific T-cell responses were assessed using IFN-ϒ ELISpot and intracellular cytokine (ICCS) assays.

Results: Vaccination generated very high magnitude HBV specific T-cell responses to all HBV antigens (Figure 1b). The mean magnitude of total HBV-specific T-cell responses in inbred BALB/c and outbred CD1 mice were 3858 and 3821 spot forming units [SFU]/10[6] splenocytes (shown on the left side of Figure 1) and 4514 and 2979 SFU/10[6] intrahepatic lymphocytes (shown on the right side of Figure 1b) respectively. ICCS showed that HBV specific CD8+ T cells were poly-functional producing both IFN-ϒ and TNF-α.

The inclusion of shark invariant chain (data shown in blue coloured bars in Figure 1b) significantly enhances the T-cell magnitude for both splenocytes (3821 mean total SFU/10[6] with sIi vs. 386 mean total SFU/10[6] without sIi, p < 0.0001) and intrahepatic lymphocytes (2979 mean total SFU/10[6] with sIi vs. 461 mean total SFU/10[6] without Sli, p = 0.0002). Importantly, T cells to the non-HBV Sli peptides were not generated.

Conclusion: We have generated a highly potent HBV vaccine that induces T-cells against all major HBV proteins, using chimpanzee

THU-475

RIPK1 depletion exacerbates progression of liver fibrosis in high fat diet induced non-alcoholic steatohepatitis (NASH) in mice

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Background and Aims: Non-alcoholic steatohepatitis (NASH) is an emergent chronic liver disease with a high prevalence in occidental countries. NASH can further progress into liver fibrosis, cirrhosis and hepatocellular carcinoma. Hepatocyte death carried through different death ligands plays key role in fibrotic progression. Previously, we showed that RIPK1, a protein kinase known to be involved in pathways related to both cell survival and death, exhibits a protective role in TNFα and FasL-induced hepatocyte death. Our new study aimed to investigate the role of RIPK1 in NASH.

Method: To decipher the role of RIPK1 in NASH, we took advantage of RIPK1^{LPC-KO} mice which are deficient for RIPK1 only in liver parenchymal cells. NASH was induced by feeding both RIPK1^{LPC-KO} animals and their WT (RIPK1$^{fl/fl}$) littermates with High Fat Diet (HFD). Mice were slaughtered at 3, 5 and 12 weeks of HFD feeding and were compared to mice fed with normal chow diet. Cytometry was performed to analyze the recruitment of inflammatory cells during the course of NASH. Plasma cytokine levels were measured by LEGENDplex^{TM}. Liver damage was assessed by histological staining and serum transaminase dosages. Liver fibrosis was quantified after Sirius red labelling.

Results: Irrespective of their genotype, mice fed with HFD, showed elevated levels of ALT and liver to body weight ratio during the development of NASH. Important liver infiltrations of immune cells, including NKT cells, were detected at week 3 of HFD. Then, after 5 and 12 weeks of treatment, the number of total immune cells decreased with a total loss of NKT cells. However, some cells such as macrophages and cytotoxic lymphocytes remained over-represented. This inflammation and immune cell infiltration were accompanied by higher plasma doses of cytokines such as TNFα and CCL2. IL-27 and IFNγ were specifically increased at week 3 of HFD. Despite similar inflammatory responses, more fibrosis was significantly evidenced at week 12 in RIPK1^{LPC-KO} as compared to their WT littermates. These findings were further supported by more elevated mRNA expression of TGFBi and TIMP2 (genes indicative of fibrosis) in RIPK1^{LPC-KO} compared to WT mice.

Conclusion: Our results show that RIPK1 in hepatocyte limits the progression of liver fibrosis during NASH.

THU-477

Clinical-grade human liver mesenchymal stem cells reduce NAS score and fibrosis progression in advanced stage NASH pre-clinical model through immunomodulation

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Background and Aims: Nonalcoholic steatohepatitis (NASH), a severe form of nonalcoholic liver diseases (NAFLD), is one of the prominent liver diseases worldwide. There is currently no approved drug for its treatment and liver transplantation is the only therapeutic approach for advanced NASH. Mesenchymal stem cells (MSCs) are promising candidates to modulate the pro-inflammatory and pro-fibrogenic environment of chronic liver because of their immuno-modulatory properties. HepaStem, adult human liver-derived MSCs isolated from organs unsuitable for transplantation can be GMP-manufactured, cryopreserved and reconstituted at the bedside as an off-the-shelf product. We previously showed that HepaStem can significantly reduce NAS score and collagen deposition in early stage NASH STAM™ model with necropsy at week 9 (Gellynck K *et al.* 2016). Safety and tolerability have been shown in a phase I/II clinical trial in

patients with metabolic disorders. The aim of this study is to evaluate the efficacy of HepaStem in an advanced stage NASH pre-clinical model.

Method: The preclinical NASH mouse model was induced by 2 hits such as streptozotocin at 2 days of age and high-fat diet from 4 weeks of age (first cell infusion at week 10 and necropsy at week 13 STAM™ model). The potency of HepaStem was compared to a vehicle co-administered with immunosuppression (cyclosporine) in order to reduce the potential xenogenic response of the recipient to human cells. As outcome, a NAFLD activity score system, which contains the steatosis, inflammation and ballooning scores, was evaluated.

Results: In an advanced stage NASH model, cell-based treatment (1 and 3 IV injections 12.5×10^6 cells/kg) significantly and dose-dependently decreased NAFLD activity score (22.4% vs 32.6% reduction, p < 0.05 and p < 0.001, respectively), which was mainly attributed to a significant reduction in inflammation and thus supporting the proposed mechanism of action (Inflammation score: vehicle vs 3 doses of HepaStem: p < 0.01). In addition, HepaStem (1 and 3 IV injections 12.5×10^6 cells/kg) cell-based treatment tended to reduce the fibrotic area thus suggesting an inhibition of disease progression in a more advanced NASH model.

Conclusion: In line with our previous results (Gellynck *et al.* 2016), we confirm that clinical grade liver progenitor cells have anti-NASH and may have anti-fibrosis effects in an advanced pre-clinical NASH model. This observation provides significant evidences to open new phase I/II studies including also more severe NASH patients as well as to apply MSCs for the treatment of chronic liver disorders.

Reference

Gellynck K, Rommelaere G, Najimi M, Tchelingerian J, Lombard C, Thonnard J, Mazza G, Sokal E. Clinical-grade human liver mesenchymal stem cells for the treatment of NASH-Fibrosis through immunomodulation. Presented at American Association for the Study of Liver Diseases, November 11–15, 2016, Boston, MA.

THU-478

Ablation of Interleukin-4 Receptor alpha in macrophages ameliorated steatohepatitis and fibrosis in murine model of non alcoholic steatohepatitis (NASH)

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Background and Aims: The role of immune cell populations in NASH-related inflammation and fibrosis remains controversial. However, targeting of specific receptors that switch immune cell phenotypes is attractive. We aimed to study the role IL-4 receptor alpha (IL-4Rα) which represents a central switch to generate Th2 T cells and M2 macrophages upon stimulation with interleukin (IL)-4 and IL-13 in a representative mouse model of NASH.

Method: 8-week-old male Balb/C wild type mice and Balb/C mice with general or macrophage specific deletion of IL-4Rα (Balb/C IL-4R$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$) mice were fed a choline-deficient, ʟ-amino acid-defined (CDAA) diet for an additional 12 weeks. Thereafter mice were sacrificed, and liver tissues sections were snap frozen and fixed in paraformaldehyde for further analysis.

Results: There was significant decrease in the liver weights of both the KO strains (Balb/C IL-4R$^{-/-}$ and LysM^{cre}IL-4Rα$^{-/lox}$) compare to the wild type mice. H&E and Sirius Red stained sections revealed a significant reduction of the NAS score (adapted for mice) from 7 to 5 and extent of fibrosis in Balb/C IL-4R$^{-/-}$ B and Balb/C LysM^{cre}IL-4Rα$^{-/lox}$ mice compared to the wild type controls. Hydroxyproline assay revealed significant reduction of fibrosis in CDAA fed IL-4R KO mice (both general and myeloid specific) compare to the CDAA fed

playing its hepatoprotective role studying WISP1 knock out (KO) mice.

Method: To establish the role of WISP1 in acute liver damage we used a knockout mouse model and we performed a time course after CCl_4 intoxication (460 mg/kg, i.p.) by collecting liver tissue at 30 min, 2 h, 8 h, 12 h, 18 h, and day 1. Damage was analyzed by necrotic area in hematoxylin & eosin staining and by transaminase activity. Different signal pathways were asses by western blot and the inflammatory state by real-time PCR of different cytokines and by immunostaining to detect immune cell infiltration. Re-introduction of WISP1 in KO mice was done by using a recombinant adeno-associated viral vector serotype 8. Different liver cell isolation was done using magnetic beads and macrophage depletion was performed by clodronate liposomes.

Results: Deletion of WISP1 led to a significant increase of necrotic area at d1 reaching 49% in KO vs 27% in WT mice. Importantly WT and WISP1 KO mice showed no significant differences in CYP2E1 expression, suggesting that the difference in tissue injury does not depend on alterations of CCl_4 metabolism in KO mice. Western blot analysis showed marked alterations in signal transduction pathways in liver tissue of WISP1 KO mice, with stronger activations of p-JNK at 2 h and p-STAT3 at 18 h and day 1. Furthermore, deletion of WISP1 leads to higher expression of inflammatory cytokines such us TNF and IL-6 and immune cell infiltration at 18h. Re-expression of WISP1 in WISP1 KO hepatocytes did not rescue the phenotype maybe due to the non-detectable WISP1 secretion in plasma. Liver cells isolation and WISP1 expression analysis showed a higher expression of WISP1 in liver sinusoidal endothelial cells and Kupffer cells compared to hepatocytes, supporting that hepatocytes are not the main source of WISP1 and non-parenchymal cells may play an important role in WISP1 expression-function in liver damage.

Conclusion: In conclusion, WISP1 is an interesting novel target in acute liver damage but further investigation is still need it.

SAT-024
Ethanol dampens IL-37 expression in liver tissue

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Background and Aims: Hepatic inflammation is a driving force in alcoholic liver disease (ALD). Interleukin-37 (IL-37) is an anti-inflammatory member of the IL-1 family. It was described that IL-37 exerts this anti-inflammatory effects in hepatic diseases, however it is not known if IL-37 has an impact on ALD. In this study, we addressed the role of IL-37 in ALD.

Method: To address if IL-37 plays a role in the development of ALD we used IL-37 expressing transgenic mice and human recombinant IL-37 in different models of alcoholic liver disease, additionally *IL-37* expression was measured in liver samples of patients suffering from alcoholic steatohepatitis and of non-alcoholic fatty liver disease patients.
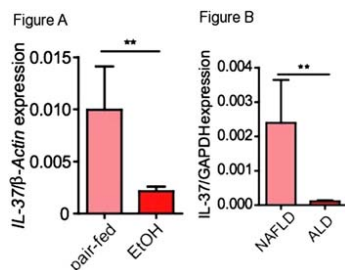
Figure A   Figure B

Results: IL-37 tg mice are not protected against hepatic injury and inflammation in experimental models of ALD. *IL-37* expression was suppressed in IL-37 transgenic mice (Figure A). Patients with ASH exhibited similarly reduced *IL-37* expression when compared to NAFLD patients (Fig. B). In a murine binge drinking model of ALD human recombinant IL-37 ameliorated hepatic inflammation.

Conclusion: We provide evidence for an exogenous noxae that suppresses Interleukin 37 expression which limits its anti-inflammatory effects in alcoholic liver disease.

SAT-025
Serum CXCL14 chemokine as a biomarker during murine acute and chronic hepatitis

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Background and Aims: Inflammation of liver is characterised by immune cells recruitment and release of chemokines. CXCL14, also called BRAK (breast and kidney cancer), is a chemokine poorly studied due to lack of knowledge of its specific receptor. In hepatic field, an up-regulation of CXCL14 mRNA has been reported in CCL4-induced liver injury in mice. However no information about the CXCL4 protein is announced. We aim to study the expression of CXCL14 protein in hepatitis developing murine models.

Method: We develop various *in vivo* models in C57Bl/6 mice: Concanavalin A-induced and murine hepatitis virus type 3 (MHV-3)-induced acute hepatitis, CCL4-induced chronic hepatitis and High Fat Diet-Induced chronic non-alcoholic steatohepatitis (NASH). Furthermore, we perform primary mouse hepatocytes (PMH) culture infected or not by MHV3 during 4 hours. ELISA, RT-qPCR, AST/ALT measurements, histology have been performed. Comparisons of parameters between two different groups were analysed by the Student t-test.

Results: Level of CXCL14 in sera of mice with acute and chronic hepatitis is increased in comparison with untreated mice. This is correlated with the increase of AST/ALT. In acute hepatitis induced by MHV3 infection, plasmatic concentration of CXCL14 is $\approx 10\,000$ pg/mL at 72 hours post-infection. Same results are observed in other acute hepatitis model as ConA administration. In chronic CCL4-induced hepatitis, serum CXCL14 concentration is ≈ 1000 pg/mL and remains constant during treatment. Beside, level of CXCL14 is not correlated with the progression of fibrosis, as observed by Sirius red labelling.

We found that infection of primary mouse hepatocytes by MHV3 did not induce CXCL14 mRNA expression but the lysis of MHV3 infected PMH allows a release of CXCL14 in conditioned media at 48 hours post-infection.

Conclusion: Our *in vivo* data show that CXCL14 chemokine is released in sera during acute and chronic hepatitis in mice. We show that hepatocytes could be a source of CXCL14 since virus-infected hepatocytes release CXCL14. CXCL14 is therefore a new biomarker linked to hepatitis, and could be involved in the development of the liver diseases.

SAT-026
Autophagy-related liver enzymes in chronic liver disease

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Background and Aims: Autophagy, the fusion of phagosomes with lysosomes is an important mechanism in hepatic homeostasis. Abnormalities of this process are associated with liver diseases,

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Titre : Rôle de RIPK1 dans la survie et la mort des hépatocytes: son implication dans des modèles d'hépatites murines.

Mots clés : Foie, Hepatite, Mort cellulaire, Inflammation, Cytokine

Résumé : La mort cellulaire joue un rôle central dans le développement et la progression des maladies du foie. Quel qu'en soit l'agent étiologique, il en résulte une destruction des hépatocytes, conduisant à une inflammation et à une prolifération compensatoire. En outre, la disparition continue de cellules peut aboutir au développement d'une fibrose, d'une cirrhose, voire d'un carcinome hépatocellulaire. L'expression ou la sécrétion de ligands de mort par les cellules inflammatoires constitue le principal facteur de la progression des maladies du foie. En aval des récepteurs de ces ligands de mort ou de TLRs, RIPK1 influence le destin de la cellule, que ce soit pour survivre ou pour mourir. Elle pourrait donc constituer une cible thérapeutique potentielle pour réguler le destin des hépatocytes. Dans cette optique, nous avons déjà montré le rôle protecteur de RIPK1 dans des modèles animaux d'hépatite aiguë induite par la ConA et le LPS. Au cours de mon travail de thèse, l'objectif était d'évaluer le rôle de RIPK1 dans des modèles murins d'hépatites aiguës (hépatite virale fulminante, dommages au foie induits par le CCl4 ou l'acétaminophène [APAP]) et d'hépatites chroniques (NASH). Nos résultats ont démontré que RIPK1 protège les hépatocytes du TNF-α sécrété par les macrophages au cours de l'hépatite fulminante induite par le virus MHV3. En outre, nous avons établi que RIPK1 dans les cellules parenchymateuses du foie n'influence pas les lésions hépatiques induites par l'APAP chez la souris. L'inhibition supplémentaire de l'activité kinase de RIPK1 chez les souris *Ripk1*LPC-KO n'a pas limitée les dommages hépatiques, révélant que l'activité kinase de la RIPK1 dans les cellules hépatiques non-parenchymateuses ne contribue pas aux lésions hépatiques induites par l'APAP. Sinon, nous avons démontré que RIPK1 dans les cellules parenchymateuses du foie préserve partiellement le foie des lésions induites par le CCl4, lésions ne dépendant pas du TNF-α.

Enfin, nous avons montré que RIPK1 dans les cellules parenchymateuses hépatiques avait tendance à limiter le développement de la fibrose induite par HFHCD dans la NASH murine et qu'une intervention alimentaire pouvait améliorer la fibrose hépatique chez la souris atteinte de NASH. Quant au rôle de l'activité kinase de la RIPK1 dans la NASH, elle reste à être explorée pour évaluer son intérêt thérapeutique.

Title: Role of RIPK1 in the survival and death of hepatocytes : its involvement in murine hepatitis models

Keywords : Liver, Hepatitis, Cell death, Inflammation, Cytokine.

Abstract : Cell death plays central role in the development and progression of liver diseases. Irrespective of the etiological agents, it results in hepatocyte destruction, leading to inflammation and compensatory proliferation. In addition, the persistent cell demise can lead into fibrosis and ultimately hepatocellular carcinoma. Expression or release of death ligands by inflammatory cells remains the key players in the progression of liver diseases. Downstream of death ligand receptors or TLRs, RIPK1 influences the fate of cell, whether to survive or to die and could therefore be potential targets in regulating cell death. In line with this, we have already shown the protective role of RIPK1 in animal models of acute hepatitis induced by ConA, LPS. In my PhD work, the objective was to assess the role of RIPK1 in animal models of acute (fulminant viral hepatitis, CCl4 and acetaminophen [APAP] induced liver damage) and chronic hepatitis (NASH). Our results demonstrated that RIPK1 protects hepatocytes from TNF-α secreted from macrophages during viral induced fulminant hepatitis. Besides, we established that RIPK1 in liver parenchymal cells does not influence APAP-induced liver injury in mice. Additional inhibition of RIPK1 kinase activity in *Ripk1*LPC-KO mice did not improve hepatic damage, revealing that RIPK1 kinase activity in liver non-parenchymal cells does not contribute to APAP-induced liver injury. Otherwise, we demonstrated that RIPK1 of liver parenchymal cells partly preserves the liver from CCl4-induced damage, lesions that do not depend on TNF-α . Finally, we showed that RIPK1 in liver parenchymal cells has a tendency to protect from HFHCD-induced fibrosis in murine NASH and that dietary intervention can improve liver fibrosis in mice with NASH. As for the role of RIPK1-kinase activity in NASH, it remains to be explored to evaluate its therapeutic interest.