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Role of pattern recognition receptors (PRR) in the pathogenesis of non-alcoholic steatohepatitis (NASH)

Doctoral thesis

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LIST OF ABBREVIATIONS

AIM2, absent in melanoma 2; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ASC, apoptosis-associated speck-like CARD-domain containing protein; AST, aspartate aminotransferase; CLR, C-type lectin receptors; CpG, cytidine-phosphate-guanosine-rich DNA; DAMP, danger associated molecular pattern; dsRNA, double stranded RNA; FFA, free fatty acid; GGT, gamma-glutamyltransferase; HCV, hepatitis C virus; HFD, high fat diet; HIV, human immunodeficiency virus; HMGB1, high mobility group box protein-1; HSC, hepatic stellate cell; IFN, interferon; IKK, IkB kinase; IL, interleukin; IPS-1, IFNβ promoter stimulator protein-1; IRAK, LI-1R-associated kinase; IRF, interferon regulatory factor; ISG, interferon-inducible gene; JNK, Jun N-terminal kinase; LMNC, liver mononuclear cell; LPS, lipopolysaccharide; LRR, leucin-rich repeats; LSEC, liver sinusoidal endothelial cells; MAVS, mitochondrial antiviral signaling protein; MCD, methionine-choline deficient; MCS, methionine-choline supplemented; MD2, myeloid differentiation factor 2; Mda5, melanoma differentiation-associated gene 5; Mult1, murine UL16-binding proteinlike transcript 1; MMP, matrix metalloproteinase; MyD88, Myeloid differentiation factor 88; NADPH, nicotinamide adenine dinucleotide phosphate; NAFLD, non-alcoholic fatty liver disease; NALP1, NACHT, LRR and PYD domains-containing protein 1; NALP3, NACHT, LRR and PYD domains-containing protein 3 / cryoporin; NASH, non-alcoholic steatohepatitis; NFkB, Nuclear factor kB; NK, natural killer cell; NLR, NOD-like receptors; NLRC4, NLR family CARD-domain containing -4; PA, palmitic acid; PAMP, pathogen associated molecular pattern; PKC, protein kinase C; Poly I:C, polyinosinic-polycytidylic acid; PSMA7, proteasome subunit alpha type 7; Rae-1α, retinoic acid early inducible-1α; RIG-I, retinoic acid-inducible gene-I; RIP3, receptor interacting protein-3; RLR, RIG-I-like receptor; ROS, reactive oxygen species; αSMA, α smooth muscle actin; TAK, TGF-β activated kinase; TBARs, thiobarbituric acid reactive substances; TBK1, TANK-binding kinase 1; TGFβ, transforming growth factor β; TIMP, tissue inhibitor metalloproteases; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; TRAF, TNFR-associated factor; TRIF, TIR domain-containing adaptor inducing IFN-beta; VISA, virus-induced signaling adaptor; zVAD, (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone)

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1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases affecting over 1/3 of the population in the Western world. The histopathology spectrum of NAFLD includes steatosis alone, steatosis with inflammation and steatohepatitis (non alcoholic steatohepatitis/NASH) that includes necroinflammation with or without fibrosis. (1). The pathogenesis of NAFLD/NASH is not fully understood yet. Recently the role of innate immunity has been implicated in the pathogenesis of NASH (1). Pattern recognition Toll-like receptors (TLR) and NOD-like receptors (NLR) are key components of the innate immune system in the recognition of pathogens, but they also sense danger signals released from damaged cells (94). The inflammation induced via the TLRs or NLRs contribute to the pathogenesis of several autoinflammatory diseases.

Fatty liver is highly sensitive to the TLR4 ligand lipopolysaccharide (LPS or endotoxin), that is a bacterial wall component of Gram-negative bacteria. Furthermore, increased plasma endotoxin levels were detected in steatohepatitis both in mice and humans. However, the role of the TLR4-MD2 receptor complex in NASH is yet to be evaluated.

Inflammasomes, large caspase-1-activating multiprotein complexes that sense both danger signals through the intracellular NLRs, are major contributors to inflammation. The NALP3 inflammasome is involved in sensing endogenous danger signals, and promotes the cleavage and maturation of the pro-inflammatory cytokine pro-IL-1β to promote/sustain inflammation. The inflammasome activation is a two step process, in which the first step usually by a TLR ligand such as LPS induce the up-regulation of the inflammasome and pro-IL-1β, and a second signal activate the inflammasome. The cell-specific expression and role of the inflammasome in the liver are yet to be evaluated in NASH.

While the factors determining progression of NASH are yet to be fully defined, the clinical importance of increased susceptibility of the fatty liver to viral infections is emerging. Co-morbidity of NASH with viral infections caused by RNA viruses, such as hepatitis C and HIV remains a clinical challenge. The pathomechanism behind the impaired antiviral immuntity is not fully clarified yet.

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2. REVIEW OF THE LITERATURE

2.1. NON-ALCOHOLIC FATTY LIVER DISEASE AND NON-ALCOHOLIC STEATOHEPATITIS

2.1.1. Definition

Non alcoholic fatty liver disease (NAFLD) is a chronic liver disease with wide spectrum of hepatic abnormalities somewhat similar to alcoholic liver disease but without significant alcohol consumption. Along with obesity, hypertension, hypercholesterolemia, hyperuricaemia and insulin resistance, NAFLD is the part of the metabolic syndrome. The histopatological spectrum of NAFLD includes steatosis alone, steatosis with inflammation and steatohepatitis (NASH) with necroinflammation (with or without fibrosis). The last form, which is progressive, can lead to cirrhosis and even to hepatocellular carcinoma (1).

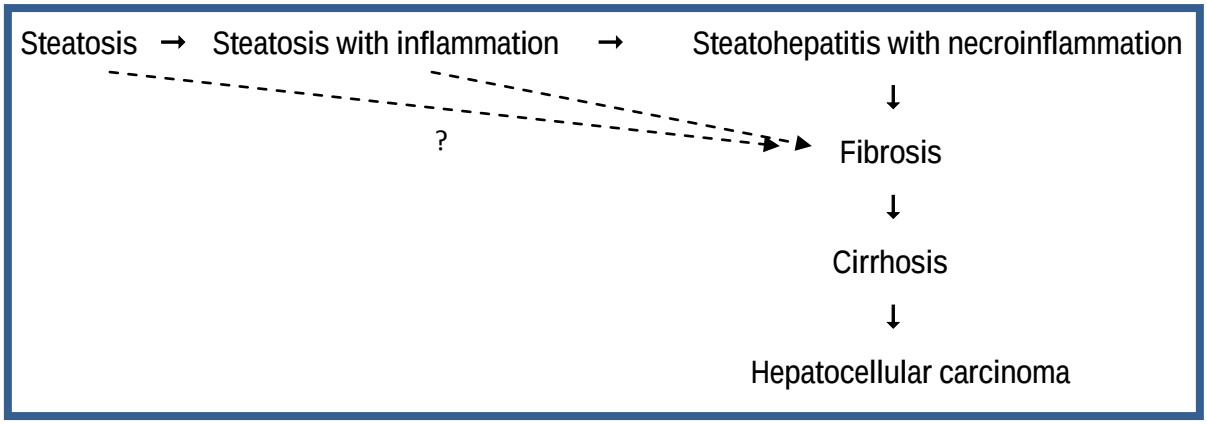

Figure 1. *Natural history of NAFLD*

2.1.2. Epidemiology

Since its first description in 1980 (2), non-alcoholic fatty liver disease (NAFLD) became one of the most common liver diseases in the Western world (3) due to the increasing prevalence of obesity. The prevalence of obesity is shown in Figure 2 and 3.

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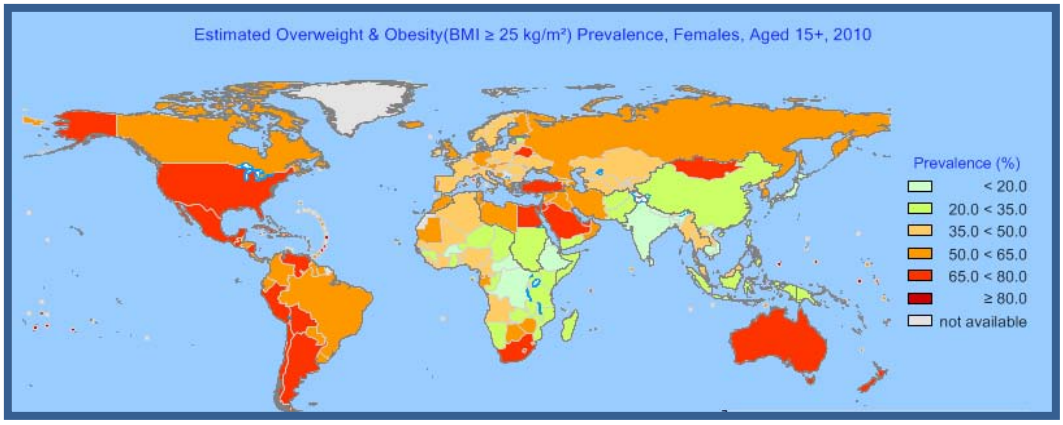

Figure 2. *Prevalence of obesity in adult females in 2010 based on the WHO Global InfoBase. (www.apps.who.int/infobase/)*

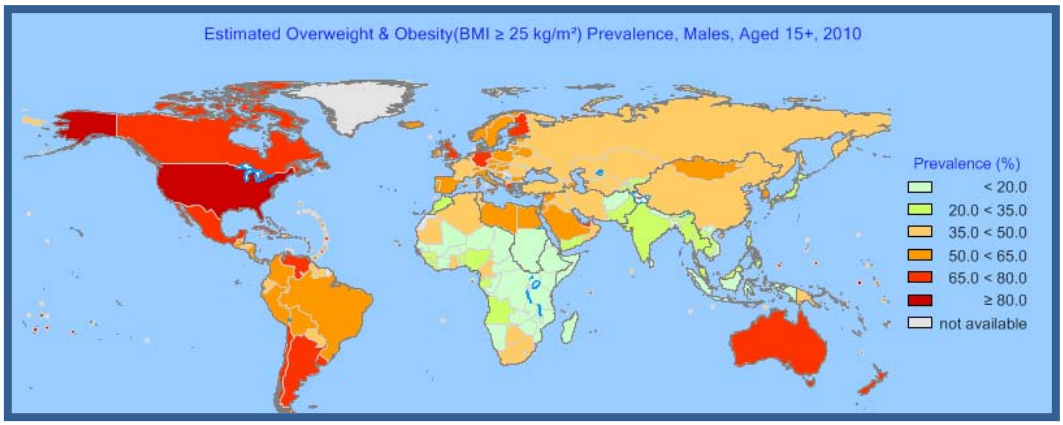

Figure 3. *Prevalence of obesity in adult males in 2010 based on the WHO Global InfoBase. (www.apps.who.int/infobase/)*

The estimated prevalence of NAFLD is around 20-30% in the general population in the Western world (4), while its prevalence is over 90% among obese subjects (5). Non-alcoholic steatohepatitis (NASH) occurs approximately in 2-3% of the general population (6), and its prevalence in the morbidly obese subjects is ~37% (5).

Notably, NAFLD affects not only the adults, but its prevalence is also increasing in the childhood: ~3% overall and ~50% in obese children (7).

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2.1.3. Clinical aspects of NASH: symptoms, diagnosis and treatment

Most of the patients with NAFLD are asymptomatic and the diagnosis is incidental during either routine blood tests (elevated liver enzymes) or ultrasound examination (liver steatosis). Fatigue, partially due to the accompanying sleep apnea and right upper quadrant abdominal discomfort due to the hepatomegaly may occur. Later, with the progression of the disease, myriad of signs and symptoms of chronic liver disease, cirrhosis and liver failure develop. In addition, since NAFLD is the "hepatic manifestation" of metabolic syndrome, these patients often suffers from non-liver related symptoms of the co-morbidities, such as type II diabetes, hypertension etc.

Increased serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT) and sometimes alkaline phosphatase (ALP) levels with exclusion of other etiologies of chronic liver diseases, such as alcohol, virus, metabolic diseases, raise the possibility of NASH (8). However, in some histologically proven NASH patients normal ALT values can be observed (9). Higher serum ferritin levels were also reported in 50% of NASH patients (10). In case of suspicion of NASH, ultrasound is used to detect hepatic fat deposition in form of hyperechogenic liver (11).

The most emerging question is to differentiate the simple steatosis from the progressive steatohepatitis using non-invasive techniques. However, to date there are no available diagnostic laboratory tests that can reliably differentiate steatosis from steatohepatitis. The measurement of keratin-18 fragments as marker of apoptosis correlates with the severity of liver disease (12) therefore may help in the staging. There are several scoring systems that might be useful in the diagnostic algorithm of NAFLD/NASH (13,14,15), including BARD score (16), NAFLD fibrosis score (17), FIB-4 score (13), APRI (AST to platelet ratio index) (18), ALT/AST ratio (13), FibroTest (19), ELF (European Liver Fibrosis) test (20), Fibroscan (21), SteatoTest (22), NASHTest (23), but they are not routinely used yet in the clinical practice (24).

To date, liver biopsy remains the gold standard of the diagnosis of NASH (24,25). The morphological signs of NASH are the followings: predominantly macrovesicular steatosis (mild: <33%, moderate: 33-66%, severe: >66%); inflammatory cell infiltration in form of portal inflammation, interface hepatitis, lobular inflammation and/or confluent

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necrosis involving monocytes-macrophages, neutrophils and lymphocytes; hepatocyte ballooning; and Mallory-Denk bodies. As the disease progresses fibrosis appears, first in the perivenular and perisinusoidal area, then portal fibrosis and bridging formation occurs that finally leads to cirrhosis (24). To evaluate the histological findings scoring systems (Brunt score, NAFLD activity score, Ishak[Knodell] score) has been developed that allows semiquantitative assessment (26, 24,27).

Besides the lack of sensitive and specific diagnostic markers that makes the diagnosis and the accurate evaluation of prevalence difficult, the optimal treatment is also still awaited. However, the enormous effort in the NASH research field to better understand the pathogenesis of the disease brings closer to the development of efficient, specific and targeted therapy. The recent knowledge about the NASH pathogenesis will be discussed below in chapter 2.1.5.

Since, insulin resistance is a key feature of NASH, dietary and lifestyle modifications that improve insulin sensitivity are the first steps in the management of NASH (28), although the long-term efficacy is questionable mostly because of the lack of compliance (29). When the body mass index (BMI) of the patient reaches 35-40, bariatric surgery can be performed to help the patients in the weight-loss, but there are no randomized controlled trials to judge the benefit-risk ratio (29). As pharmacologic treatment the followings are used: insulin sensitizers (metformin, pioglitazone, rosiglitazone); antioxidants and hepatoprotectants (Vitamin E, Silymarin [30]); ursodeoxycholic acid (UDCA [31]) and TNFα antagonists (Pentoxyfilline [32]) (29). Among the insulin sensitizers, pioglitazone has been reported to be the most efficient (33,34), but none of them improved the fibrosis convincingly, and the discontinuation of the drug resulted in the re-elevation of the liver enzymes (35). The antioxidant Vitamin E alone or in combination with pioglitazone is efficient and recommended (36) but only in selected patient population. Several other drugs have been tried in the treatment of NASH in animal experiments and clinical trials including glucagon-like peptide 1 (GLP-1) (exenatide) (37), betaine (38), angiotensin receptor blockers (39), endocannabinoid antagonist rimonabant (40), lipid lowering fibrates and statins (41), but there is no final conclusion and consensus about their use in the NASH therapy.

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2.1.4. Fatty liver and viral infections

While the factors determining progression of NASH are yet to be fully defined, the clinical importance of increased susceptibility of the fatty liver to ischemia (42,43), bacterial lipopolysaccharide (LPS) (44), and drug-induced (45,46) liver damage is emerging.

Co-morbidity of NASH with RNA viral infections, such as hepatitis C and HIV remains a clinical challenge (47). HCV-infected patients with significant steatosis or superimposed NASH have rapid progression of liver disease, increased rate of fibrosis, and a decreased likelihood of sustained virological response (SVR) to standard antiviral therapy (48). Patients with chronic HCV infection exhibit high prevalence of metabolic abnormalities (49,50), while HCV clearance by sustained virological response ameliorates insulin resistance (48,50). In human immunodeficiency virus (HIV) infection highly active anti-retroviral therapy (HAART) induces extensive alterations to liver lipid metabolism, including liver damage and sometimes even liver failure (51,52,53,54,55). Fatty liver also may complicate viral infections, such as hepatitis A virus (HAV)-, cytomegalovirus (CMV)-, or Epstein-Barr virus (EBV) resulting in “acute on chronic” liver failure.

The susceptibility of fatty liver to virus-induced liver damage urges the better understanding of changes of antiviral immune responses in steatotic livers.

2.1.5. Pathogenesis

In 1998, the “two-hit hypothesis” of NASH pathogenesis was proposed in which the initial step (1^{st} hit) involves fat accumulation in the liver as a result of excessive delivery of free fatty acids (FFA) from the adipose tissue, and imbalance of lipid synthesis and export in hepatocytes (56). The steatosis then increases the susceptibility of the liver to “2^{nd} hits”, such as oxidative stress, gut-derived bacterial endotoxin, inflammatory cytokines/adipokines etc. which in turn lead to the progression of the disease, steatohepatitis and fibrosis (57). However, challenging this dogma, there are increasing evidences that free fatty acids (FFA) can directly cause liver injury and inflammation without additional second hits (58,59).

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Recently, a “multiple parallel hits” model was proposed by *Tilg H et al.* where they even question the order of steatosis-inflammation, and suggest that in some cases of NASH inflammation may precede steatosis and itself lead to fat accumulation (60).

Insulin resistance, one of the key factors of NAFLD pathogenesis, lead to impairment of β-oxidation of FFAs (61) and also impairment of suppression of adipose tissue lipolysis (62) thus promoting hepatic lipid accumulation. Vice versa, steatosis can cause insulin resistance by activation of serine kinases including Jun N-terminal kinase (JNK), inhibitor of nuclear factor κB (IKK) and protein kinase C (PKC) (63). Furthermore, insulin resistance may augment the inflammation in NASH (64).

Beyond the insulin resistance, increasing evidence suggests the crucial role of innate immune system in the NASH pathogenesis (65,66), and innate immunity is also important in the development of insulin resistance (67). The pro-inflammatory cytokines TNF-α and IL-6 are involved in the progression of NALFD (68). Increased serum and liver TNF-α levels were observed in NASH patients, that correlated with the severity of the disease (69,70,71). The increased serum IL-6 levels in obese patients reduced with weight loss (72) and its pathogenic role was reported in high fat diet induced hepatic steatosis and insulin resistance (73), although contradictory studies exist as well (74).

The pro-inflammatory cytokines can be produced in the liver, but also can be secreted by the adipocytes and the liver might be the main target for adipose tissue derived TNF-α and IL-6 (60). Obesity affects the macrophage recruitment and also macrophage phenotype in the adipose tissue (65). Other adipocyte-derived cytokines (adipokines) that has been investigated in the NASH pathogenesis are the leptin and adiponectin. Higher leptin and lower adiponectin levels were reported in patients with NAFLD (75,76). Although there are high leptin levels, but due to receptor disorders, leptin resistance was observed (77). In contrary, adiponectin is an anti-inflammatory cytokine and high level of adiponectin diminishes hepatic steatosis (78).

Not only the adipose tissue, but the gut-microbiota contributes too to the development of steatohepatitis (79). Endotoxin or lipopolysaccharide (LPS), a bacterial wall component was suggested as a “2nd hit”, resulting in progressive liver injury (56). Increased plasma endotoxin levels were detected in mice with steatohepatitis (80), and in

humans with NAFLD (81). Importantly, fatty liver is highly sensitive to LPS and the deficiency of its receptor attenuates hepatic steatosis and inflammation in an animal model of NASH (82). The fact that probiotics might be useful in the therapy of steatohepatitis (83,84) support that gut microbiota has significant influence on the systemic immune responses. Furthermore, deficiency of Toll-like receptor 5 (TLR5) affect the composition of gut microbiota and lead to the development of metabolic syndrome (85). The role of TLRs in liver disease, particulary in NASH, will be discussed in chapter 2.2.3.

As subcellular localizations of the pathologic events, the role of both the mitochondria and the endoplasmatic reticulum is well-established. Structural and functional abnormalities of the mitochondria were reported (86,87) in NASH patients as well as in animal models of steatohepatitis (88). Mitochondria is one of the main sources of reactive oxygen species (ROS) and therefore it is significant contributor the oxidative stress observed in NASH (89). Several factors, including saturated FFAs can induce endoplasmatic reticulum (ER) stress (90) that leads to inflammation. An ER-related process, autophagy has evolved recently as another pathway involved in steatohepatitis (91).

Finally, genetic factors such as patatin-like phospholipase 3 (PNPLA3) (92) might also help to explain why some patients have progressive steatohepatitis while others not (60).

The two-hit hypothesis and the multiple hypothesis models are shown as Figure 4 and Figure 5.

The knowledge about the pathogenesis of NASH is continuously broadening, but it is not fully clarified yet.

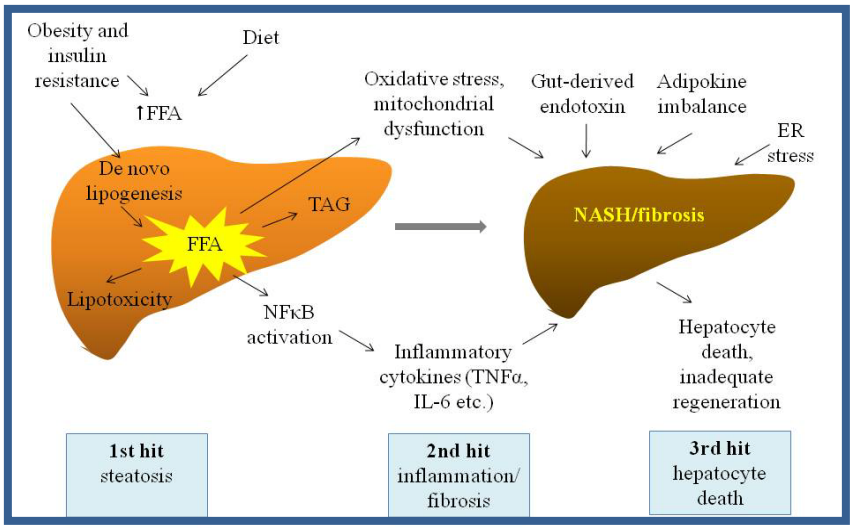
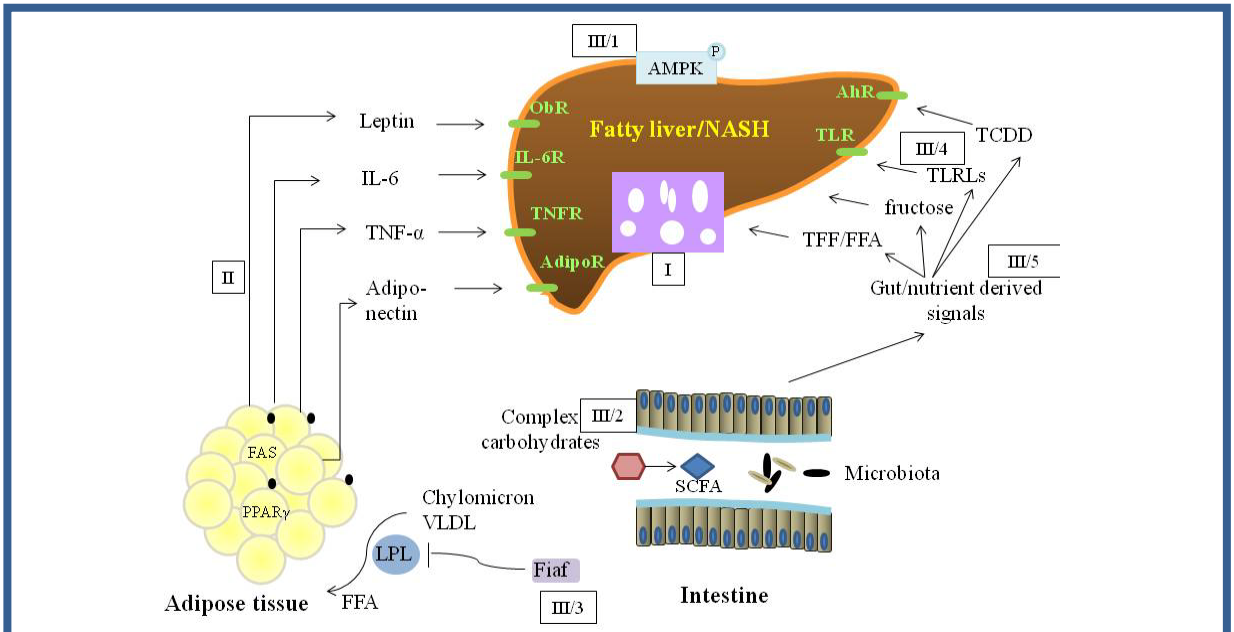


Figure 4. *The "modified two hit hypothesis model" of NASH pathogenesis. The figure is based on the publication of Dowman JK et al., Q J Med 2010;103: 71-83.(93)*



I. *Liver loaded with lipids;* ***II.*** *Adipose tissue: Adipokines (leptin, adiponectin, TNF-α, IL-6); PPARγ; FAS;* ***III.*** *Gut:* ***1.*** *Absence of microbiota lead to increased activity of phosphorylated AMPK,* ***2.*** *Complex carbohydrates metabolized to SCFAs (proprionate, acetate), Gpr41 and Gpr43 ligands, shortage of SCFAs → inflammation,* ***3.*** *Decreased (by microbiote) fasting induced adipocyte factor (Fiaf), a circulating lipoprotein lipase (LPL) inhibitor,* ***4.*** *TLR (toll-like receptor) ligands (TLR5, TLR9, TLR4),* ***5.*** *Nutrients: trans fatty acids (TFA); fructose; 2,3,7,8-tetrachlorodibenzodioxin (TCDD, AhR [aryl hydrocarbon receptor] ligand)*

Figure 5. *The "multiple parallel hits model" of NASH pathogenesis. The figure is based on the publication of Tilg H & Moschen AR, Hepatology 2010;52: 1836-1846.*

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2.2. PATTERN RECOGNITION RECEPTORS (PRRs)

As mentioned above, both bacterial components and danger signals released from damaged cells plays crucial role in the pathogenesis of NASH. Pattern recognition receptors including toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) are the initial sensors of infections/PAMPs (pathogen-associated molecular patterns) and endogenous danger signals/DAMPs (danger associated molecular patterns). TLRs and CLRs are transmembrane proteins, while RLRs and NLRs are cytoplasmic sensors expressed by both immune and non-immune cells (94). The activation of PRRs by their ligands induces the transcription of several genes involved in the innate immune responses, such as pro-inflammatory cytokines, chemokines, Type-I interferons, antimicrobial proteins and many others (94). In addition, some NLRs form multiprotein complexes called inflammasomes that are important for the maturation of some pro-inflammatory cytokines (94).

This thesis focuses on the role of TLRs and inflammasomes in the pathogenesis of NASH, and discusses the role of RLRs in terms of impaired antiviral immunity in steatohepatitis.

2.2.1 Toll-like receptors (TLRs)

2.2.1.1. TLRs and their ligands

The Toll pathway was initially identified in *Drosophila melanogaster* involved in the embryonic development (95,96). Later on it became evident that the Toll pathway involved not only during embryogenesis, but also important in the innate immunity (97). Furthermore, they were identified in other species than Drosophila as an evolutionary conserved signaling cascade (98).

They consist from an N-terminal leucine-rich repeats (LRRs), a transmembrane region and a cytoplasmic Toll/IL-1R homology (TIR) domain. To date twelve TLRs have been identified in mice and ten in humans (94).

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Figure 6 shows the TLRs with their corresponding ligands and the intracellular signaling cascades.

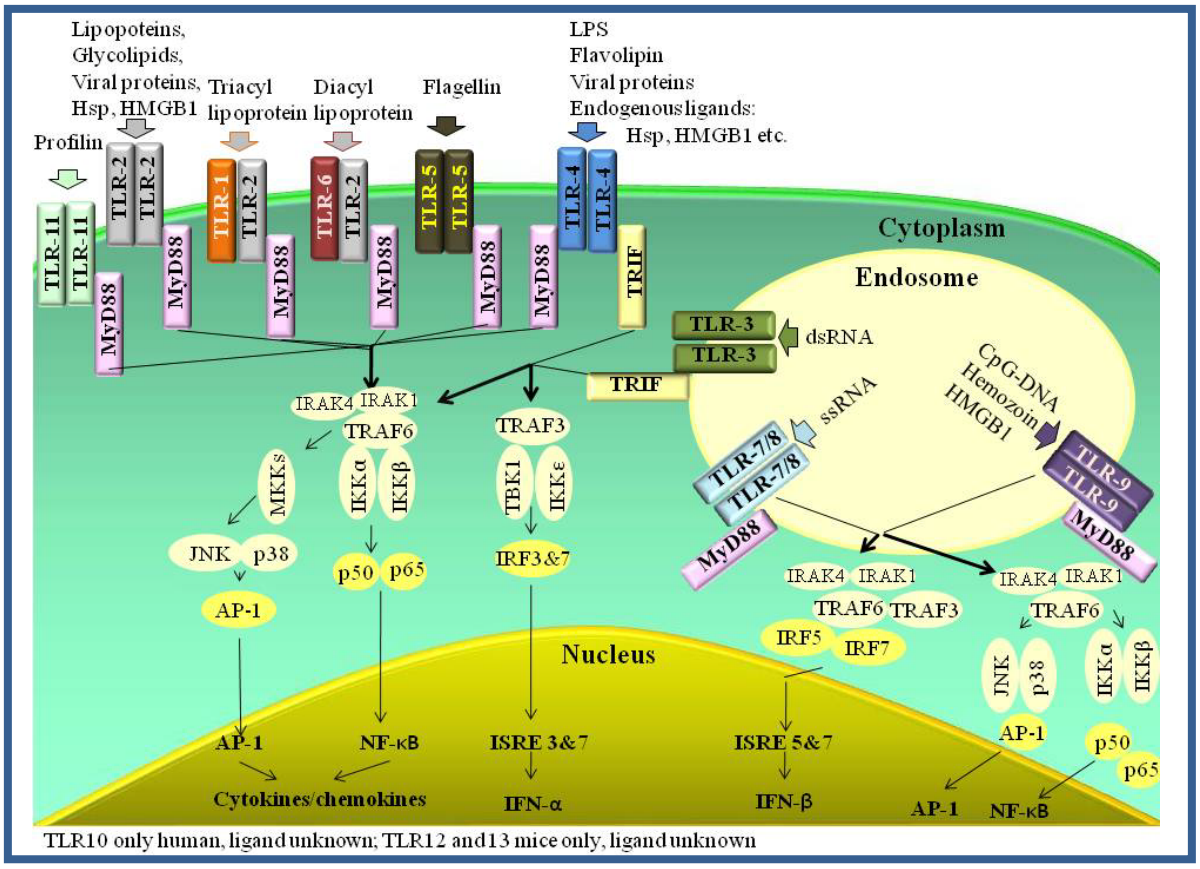

Figure 6. *Toll-like receptors, their ligands, and the TLR signaling cascade*

TLR signaling can be divided into myeloid differentiation factor 88 (MyD88)- and TIR domain-containing adaptor inducing IFN-β (TRIF)-dependent pathways that will be discussed below demonstrated on TLR4 since it triggers both MyD88- and TRIF-pathways.

TLR4 forms a receptor complex with myeloid differentiation factor 2 (MD2) on the cell surface and two TLR4-MD2 complexes create a homodimer *(Figure 7.)*. TLR4 is the main sensor of endotoxin (LPS), but beyond LPS, it also recognizes bacterial flavolipin, mannan, viral proteins (99) and endogenous danger signals, including heat shock proteins, HMGB1, hyaluronan and fibronectin fragments (100).

Ligand engagement recruits MyD88 to the receptor via TIRAP/Mal followed by an interaction between MyD88 and IL-1R-associated kinase (IRAK)-4. IRAK4 then activates

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IRAK1 that associates with TNFR-associated factor 6 (TRAF6) and lead to the activation of TGF-β-activated kinase-1 (TAK1), and TAK-1 binding protein (TAB) 1,2 and 3. That later complex phosphorylates IκB kinase (IKK) and MAP kinase (MAPK) 6. Phosphorilated IκB degrades and thus the free nucear factor-κB (NFκB) can translocate to the nucleus. MAP kinases activate another transcription factor, AP-1. NFκB and AP-1, finally, lead to the transcription of several pro-inflammatory genes (100). The MyD88 signaling induced by the endosomal TLR9 and TLR7 or 8 is somewhat different (without details depicted in *Figure 6*.) and lead to IFN-β production via IRF7, 5 or 1 (100).

TLR4-ligands may activate the TRIF pathway as well via TRIF-related adaptor molecule (TRAM). Active TRIF associates with TRAF6 and TRAF3. TRAF3 activates the kinases IKKε and TBK-1 (TANK-binding kinase 1) leading to the activation of interferon regulatory factor (IRF) 3 and 7 and Type-I interferon production. TRIF-induced TRAF6 pathway downstream is similar to MyD88-induced TRAF6 signaling resulting in pro-inflammatory cytokine and chemokine transcription. TLR3 uses only TRIF as adaptor (100).

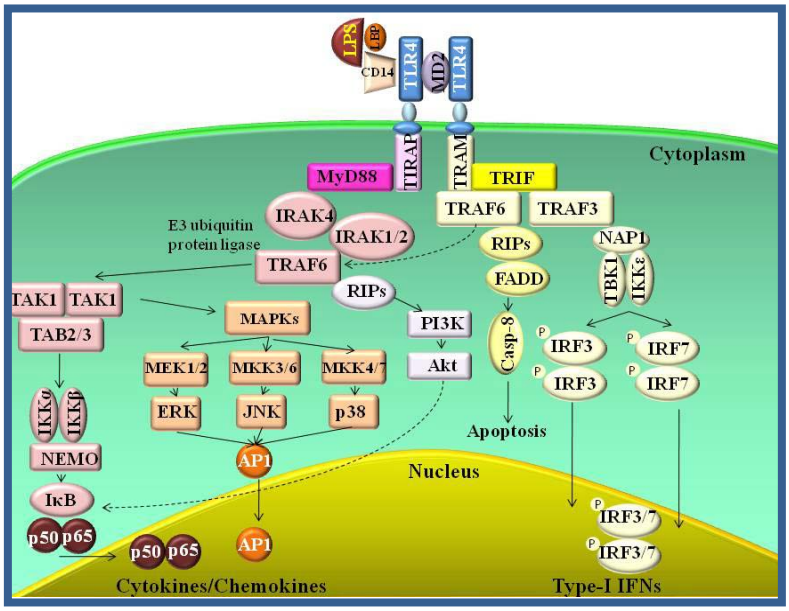

Figure 7. *Structure and downstream signaling of TLR4-MD2 complex*

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2.2.1.2. TLRs in liver diseases

Toll-like receptors are expressed in several cell types in the liver (101). The hepatic resident macrophages (Kupffer cells) express TLR-2, -3, -4 and -9 and produce pro- and anti-inflammatory cytokines in response to TLR ligands. The plamocytoid dendritic cells (pCDc) produce IFN-α and also pro-inflammatory cytokines in response to TLR7/8 and TLR9 stimulation, while conventional-DCs respond to TLR-2,-3 and-4 ligands with cytokine production in the liver. The hepatic natural killer (NK) cells express TLR-1,-2,-3,-4,-6, -7/8, -9 and those TLR-ligands together with Kupffer-cell derived IL-12 induce IFN-γ production. TLRs activate B-cell proliferation, while T-cells are rather indirectly activated by the TLRs. Beyond the immune cells, liver parenchymal cells also express TLRs. Hepatocytes although do express all TLRs, but they can respond only to TLR2 and TRL4 ligands. Liver sinusoidal endothelial cells (LSEC) respond to TLR4 ligand LPS, and hepatic steallate cells (HSCs) can be stimulated by TLR4 and TLR9 ligands. (101)

The pathogenic role of TLRs has been demonstrated in infectious (HCV, HBV, endotoxin-induced liver damage, Listeria, Salmonella and malaria infections) and non-infectious (alcoholic liver disease, NASH, ischemia-reperfusion injury, primary biliary cirrhosis, autoimmune hepatitis) liver diseases and in their complications (hepatic fibrosis, hepatocellular carcinoma). The role of TLRs in liver diseases was reviewed by *Seki et al.* (101)

2.2.2. RIG-I-like receptors (RLRs)

2.2.2.1. RLRs and their ligands

The cytoplasmic RIG-I-like helicase receptors include retinoic acid inducible protein I (RIG-I), melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). They contain a C-terminal regulatory domain, a central DEAD box helicase/ATPase domain and 2 N-terminal caspase recruitment domains (CARDs) except LGP2 that lacks the CARD domain. They have low expression, but viral infections and Type I IFNs highly enhance the RLR expression (94). Upon engagement by viral dsRNA (either genomic RNA of dsRNA viruses or dsRNA generated during the replication of ssRNA or dsRNA transcribed from dsDNA by polymerase III) RLR

signaling results in Type-I IFN and pro-inflammatory cytokine production (102,103). Although, both RIG-I and MDA5 sense viral RNA, their ligand specificity somewhat differs. RIG-I recognizes short dsRNA and the presence of 5`triphosphate increases Type I IFN induction. In contrast, MDA5 senses long dsRNA such as the synthetic poly I:C (102). RIG-I is important for the detection of Paramyxoviridae (eg. Sendai virus), Influenza A virus, Rhabdoviridae (eg. Vesicular Stomatitis Virus), Myxoma virus and Herpesviridae (eg. Ebstein Barr virus), while MDA5 is crucial for the recognition of Caliciviridae, Picornaviridae (eg. Encephalomyocarditis virus), Murine hepatitis virus and Vaccinia virus (104). Both RIG-I and MDA5 contribute in the detection of Reoviridae and Flaviviridae such as Hepatitis C virus (103). LGP2 likely cooperate with RIG-I and MDA5, since LGP2 deficiency decreases certain viruses-induced IFN production (104).

RLR stimuli lead to the interaction between the CARD domain of the RLR receptor and the CARD domain of the adaptor protein called MAVS (mitochondrial antiviral signaling protein) or IPS-1 (IFN-β-promoter stimulator 1) or VISA (virus-induced signaling adaptor) (105). The name MAVS will be used further on in that thesis. MAVS is localized in the outer membrane of the mitochondria that is crucial for the downstream signaling. Beyond the N-terminal CARD domain MAVS contains a C-terminal TM domain, that is responsible for the mitochondrial anchorage (106). In addition, *Baril M et al.* has recently shown that the TM domain is the main determinant of the self-interaction, and they demonstrated that MAVS oligomerization is essential for the downstream activation of IRF3 and NFκB (107). The TM-dependent dimerization of MAVS provides an interface for binding and activating TRAF3 (106). Similarly to the TRIF signaling pathway, TRAF3 activation results in the phosphorilation and oligomerization of IRF3 and IRF7 via TBK1 and IKKε, and the active IRF3 lead to the production of Type-I IFNs (94). MAVS also interacts with Receptor interacting protein kinase 1 (RIP1) and Fas-associated death domain protein (FADD) that lead to the complex formation with caspase-8 and 10, and to the cleavage and activation of these caspases and apoptosis. The RIP1/TRADD/FADD complex also induces inflammatory cytokine production via NFκB activation (102). The RLR signaling is shown in *Figure 8.*

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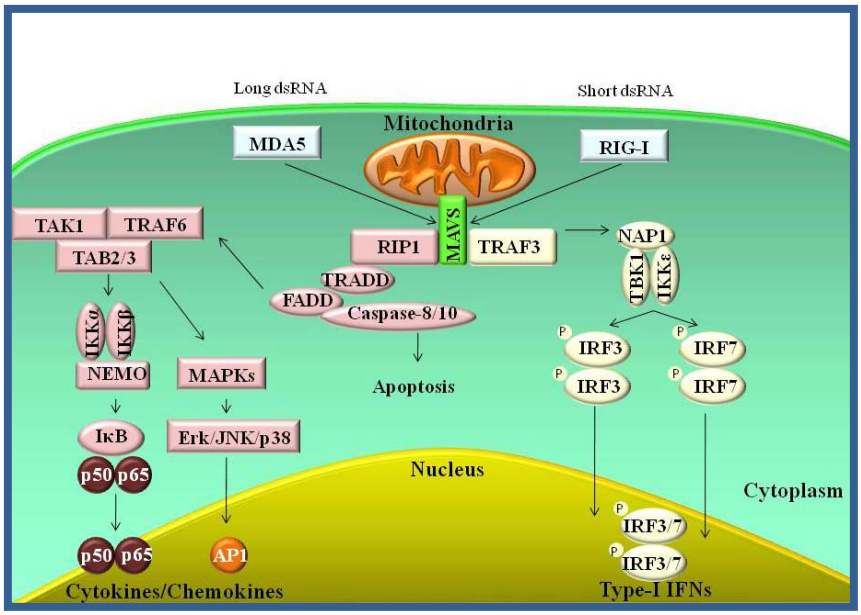

Figure 8. *RLR signaling*

2.2.2.2. RLRs in liver diseases

Helicase receptors are expressed in several cell types in the liver, including hepatocytes, conventional dendritic cells, Kupffer-cells, NK cells, endothelial cells and fibroblasts (108,109). Immunohistochemistry revealed that in naïve mice MDA5 is broadly expressed in hepatocytes and interstitial cells (Kupffer cells and endothelial cells) in the liver, but synthetic dsRNA (poly I:C) (110), as well as Type I IFNs further increase its expression.

The RLR-mediated antiviral responses are extensively studied in hepatitis C infection in the liver (111,112). RIG-I is essential for the innate immune signaling in hepatocytes triggered by HCV genome. However, HCV has evolved strategies to disrupt the host antiviral response eg. by cleaving MAVS from the mitochondria (113) by the viral NS3/4A protease. MAVS can be cleaved by other viruses too such as the 3ABC protease of hepatitis A virus (114). The target of the viral cleavage is at the C-terminal region (Q428, C508), such as in case of apoptotic cleavage (D429) (113). Beyond viral hepatitis, the role of RLRs in the pathogenesis of biliary diseases has been suggested recently (115).

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2.2.3. INFLAMMASOMES

The third big group of pattern recognition receptors is the family of Nod-like receptors (NLRs) (94). NLRs are composed from a C-terminal leucin-rich-repeat (LRR) domain that plays role in the recognition of ligands, a central NACHT (NAIP, CIITA, HET-E and TP-1) domain that is responsible for the oligomerization and dNTPase activity, and an N-terminal CARD or pyrin (PYD) domain. Based on the NACHT domain three subfamilies have been distuinguished: a) NODs, [NOD1-5, CIITA] b) NLRPs or NALPs [NLRP / NALP 1-14] and c) IPAF [IPAF, NAIP] subfamily. Several NLRs plays role in the formation of a multiprotein complex called inflammasome.

2.2.3.1. Definition

Inflammasomes are intracellular multiprotein complexes that in response to pathogens or danger molecules activate the cysteine protesase caspase-1 that in turn results in the maturation of pro-inflammatory cytokines, including IL-1β and IL-18, the proteolytic inactivation of IL-33 and furthermore they contribute to the regulation of cell survival and cell death.

2.2.3.1. Types of inflammasomes

To date four main prototypes of inflammasomes are characterized: NLRP1 (NALP1); NLRP3 (NALP3, cryporin); NLRC4 (IPAF) and the recently described AIM2. With the exception of AIM2, the nomenclature of inflammasomes is based on the NOD-like receptor (NLR) that form complex with the effector molecule pro-caspase-1 with or without the help of an adaptor molecule and lead the auto-activation of the caspase-1.

NLRP1 (NALP1), the first described inflammasome, is able to interact directly with caspase-1 due to its C-terminal CARD domain. However, the presence of ASC enhances the activity of the complex in humans. Murine NLRP1 is unable to bind to ASC because it does not contain functional PYD domain.

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NLRP3 (NALP3), the most fully characterized member of the inflammasome family, consists of the PYD, NACHT and LRR domain containing Nod-like receptor, NLRP3, the adaptor molecule ASC and the effector molecule pro-caspase-1. Since NLRP3 does not contain CARD domain, the presence of the adaptor molecule is necessary for the complex formation.

IPAF (NLRC4) also contains a CARD domain resulting in direct interaction with caspase-1, but some studies suggested the requirement of ASC for the maximal caspase-1 activation.

AIM2, is a PYD and HIN-200 domain containing protein that recruit caspase-1 via the ASC adaptor molecule, since itself is lacking the CARD domain.

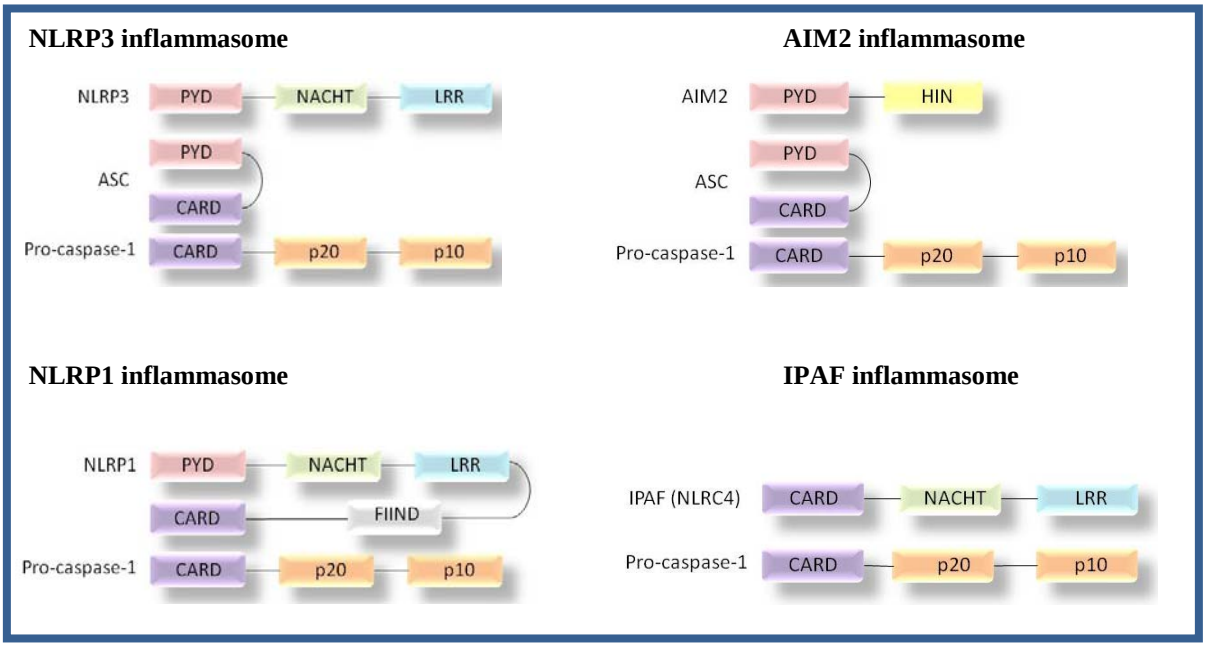

Figure 9. *Structure of the NLRP3 (NALP3), NLRP1 (NALP1), IPAF and AIM2 inflammasomes*

2.2.3.3. Function of inflammasomes

Inflammasome activation leads to auto-activation of the 45kDa inactive pro-caspase-1 precursor into p20 and p10 subunits that form the active caspase-1 (116). The cysteine protease caspase-1 belongs to the inflammatory caspases together with caspase--

11 and -12 in mice and caspase-4 and -5 in humans (117). Active caspase-1 cleaves the precursors of IL-1β and IL18 to their mature form or inactivates IL-33 (117,118).

IL-1β is a pro-inflammatory cytokine, a central regulator of inflammation that binds to IL-1 receptor (IL-1R) to exert its broad biological effects. The IL-1R also recognizes IL-1α and binds IL-1R antagonist (IL-1Ra), the latter has an inhibitory effect on the IL-1R (119). The transcription, translation and secretion of IL-1β are tightly regulated (119). IL-18 or IFN-γ inducing factor, activates Natural Killer (NK) cells to produce IFNγ (120). IL-18 precursor is constitutively expressed in human PBMCs and mouse spleen cells, but its maturation and secretion is controlled by the inflammasomes (121). IL-33 is a chromatin-associated cytokine of the IL-1 family that drives Th2 responses (122,123). The full-length active IL-33 is cleaved and inactivated by caspase-1 (118).

Beyond, the maturation of pro-inflammatory cytokines, inflammasomes activation regulates cell death. Pyroptosis, first described in *Salmonella* infected macrophages, is a caspase-1 dependent cell death showing similarities to apoptosis (DNA-damage), but it does not depend on apoptotic caspases and it is accompanied with loss of plasma membrane integrity and lack of chromatin condensation (124). NLRP1 (NALP1), NLRC4 (IPAF) and NAIP activate pyroptosis (119) while NLRP3 (NALP3) contributes to another NLR-dependent cell death, pyronecrosis. Pyronecrosis shows similarities with necrosis, since it is not caspase-depedent and leads to breakdown of plasma membrane without chromatin condensation. Pyronecrosis utilizes the inflammasome adaptor molecule, ASC, and involves the lysosomal cathepsin B (119). Both pyroptosis and pyronecrosis elicit inflammation.

Finally, we have to mention that caspase-1 can promote cell survival via SREBPs in HeLa and CHO cell lines (125).

2.2.3.4. Inflammasome activating ligands

Inflammasome activation is a 2-step process in which signal 1 results in up-regulation of inflammasome expression (mostly from TLR activation) and signal 2 triggers

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functional inflammasome activation by an inflammasome activator (116). Inflammasome activators can be pathogen-associtaed (PAMPs) or endogenous danger molecules (DAMPs) summarized in *Table 1*.

Table 1. *Known activators of inflammasome NLRs*

Inflammasome	Activator
NLRP3 (NALP3, cryoporin)	***Large particles via phagocytosis***
	Monosodium urate crystals (MSU) (126)
	CPPD (calcium pyrophosphate dehydrate) (126)
	Alum (127)
	Silica (128)
	Asbestos (129)
	Cholesterol crystals (130)
	Amyloid beta (131)
	Hyaluronan (132)
	Hemozoin (133)
	Vaccine adjuvants (poly lactide-co-glycolide and polystyrene microparticles) (134)
	Bacterial toxins (pore forming)
	Listeria monocytogenes Lysteriolysin O (135,125)
	Staphylococcus aureus alpha-toxin (135,125,136)
	Aeromonas hydrophila aerolysin (135,125)
	Streptolysin (137)
	Nigericin (135)
	Maitoxin (Dinoflegellates) (125)
	Ion channels and activators
	ATP(P2X7) (135)
	Influenza virus M2 channel protein (138)
	PAMPs **(only if transferred to the cytoplasm by eg. Streptolysin O poreformin toxin)**
	LPS, lipid A, PGN, MDP, LTA, Pam3, ssRNA, dsRNA, CpG DNA (139,140)
NLRP1 (NALP1)	Bacillus anthracis lethal toxin (141)
	MDP (142)
NLRC4 (IPAF)	***Gram negative bacteria (flagellin-dependent and independent)***
	Salmonella typhymurium (143)
	Shigella flexneri (144)
	Legionella pneumophila (145)
	Pseudomonas aeruginosa (146)
AIM2	***dsDNA***
	bacterial (147,148)
	viral (148)
	mitochondrial (149)
	host (148)

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NRLP1 (NALP1) inflammasome

To date only the bacterial wall component muramyl dipeptide (MDP) and the *Bacillus anthracis* lethal toxin has been shown to activate NRLP1 inflammasome (141,142). The exact pathomechanism has not clarified yet, but potassium efflux has been suggested to play role in the NLRP1 inflammasome activation (150,151). We have to mention that NLRP1 localize mostly in the nucleus in contrary to other inflammasomes that are cytoplasmic (152).

NLRP3 (NALP3) inflammasome

NLRP3 is the most characterized inflammasome. The activation of NLRP3 is tightly regulated at transcriptional level via NFκB (153). Cell priming with an NFκB activator such as the TLR4-ligand LPS is the first and critical step of inflammasome activation (150). Although up-regulation of NLRP3 expression is required, but not sufficient for the inflammasome activition (150). Several stimuli have been shown to serve as second signal for the activation of NLRP3 inflammasome. The exact mechanism by that the huge varieties of activators lead to the auto-activation of caspase-1 is not fully clarified yet.

To date three major pathways have been implicated in NLRP3 inflammasome activation: 1. ROS production, 2. lysosomal disintegration and 3. potassium efflux. The recent knowledge about the NLRP3 activation is summarized in *Figure 10*. Since NLRP3 inflammasome can be activated by several, not even alike ligands, the theory that those stimuli can activate the NLRP3 inflammasome via ROS production as a common pathogenic pathway is attractive. Several groups have reported that ROS scavengers suppress inflammasome activation (149,154,155,156). Mitochondria can serve as source of ROS (149), and NADPH oxidase may also contribute, since the blockage of NADPH oxidase inhibits inflammasome activation (150). Furthermore, both large particles (157) and ATP (152) induce ROS production. However, how can ROS induce inflammasome activation has not been clarified yet. Recently, the ROS-dependent release of thioredoxin-interacting protein (TXNIP) from thioredoxin and direct interaction between TXNIP and

NLRP3 has been described (153). Notably, some ligands that lead to ROS production, does not lead to inflammasome activation (158).

The second pathway of inflammasome activation is induced by crystals or large particles such as silica, asbestos, alum, amyloid, monosodium urate, cholesterol (126-134). The particles are phagocytosed and after the fusion of the phagosome and lysosome, the breakdown of the phagolysosomal membrane results in the release of lysosomal content into the cytoplasm inducing inflammasome activation. The role of cathepsin B, a lysosomal protease has been implicated in the NLRP3 activation (131). Notably, caspase-1 activation by large particles is not impaired in cathepsin B-deficient macrophages (159).

The third pathway is via potassium efflux. Extracellular ATP can stimulate the P2X7 purinergic receptor that in turn results in potassium efflux and the recruitment of pannexin. The later one is a membrane pore that allows the delivery of extracellular PAMPs and DAMPs into the cytosol (139).

NLRC4 inflammasome

NLRC4 (IPAF) inflammasome is activated by the flagellin of Gram-negative bacteria including *Salmonella typhimurium, Pseudomonas aeruginosa and Legionella pneumophila* and some Gram positive flagellated bacteria such as *Listeria monocytogenes* (143-146). On the other hand, NRLC4 can be activated by non-flagellated bacteria as well, including the Gram negative *Shigella flexneri* (144). The steps of NRLC4 inflammasome activation are not explored yet.

AIM2 inflammasome

Muruve et al. described that bacterial, viral and mammalian host DNA can trigger caspase-1 activation. Later AIM2, a HIN200 protein has been identified as a cytosolic dsDNA sensing inflammasome (147,148). It was the first description of a non-NLR family member that forms inflammasome complex and lead to caspase-1 activation. Since then, it has been shown that RIG-I also can trigger caspase-1 activation via the adaptor molecule ASC (160).

AIM2 is also unique in terms of that direct ligand binding has been proven (147). It is important in the recognition of bacterial DNA, DNA viruses and may also contribute to the pathogenesis of autoimmune diseases by recognizing mammalian DNA (161).

The characterization of other NLRs and the exact pathomechanism leading to inflammasome activation is still awaited.

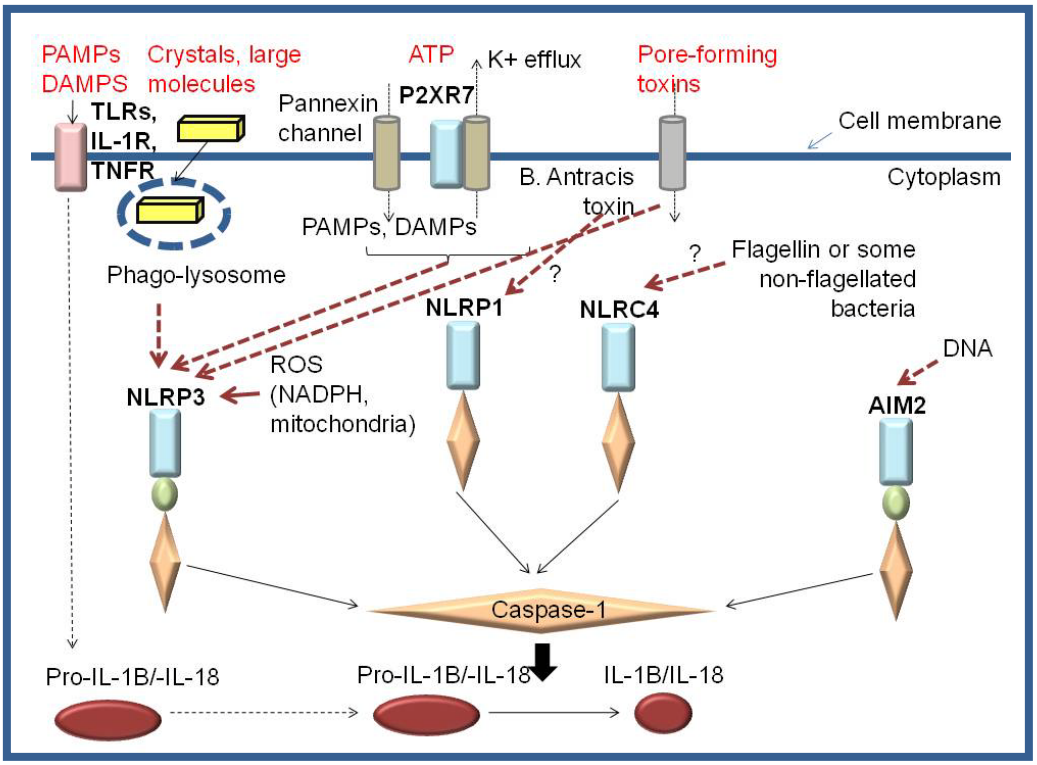

Figure 10. *Signaling transduction pathways of inflammasome activation*

2.2.3.5. Inflammasome expression in the liver

The expression of inflammasomes and subcellular localization of the different NLRs varies between tissues (159). Early studies showed highest expression of NLRP3 (CIAS1) and NLRP1 (NAC) in peripheral blood leukocytes, while the liver showed relatively low levels (162,163). The liver expresses NLRP1, 2, 3, 6, 10, 12 and NLRC4 at the mRNA levels (164). The expression of PRRs is lower in solid organs compared to

spleen likely due to the lack of higher number of splenic immune cells (164,165). However, human livers express higher level of NLRP10 (164), while murine livers are high in NLRP6 expression (164) compared to the spleen. The significance of these NLRs is yet to be evaluated.

The liver is comprised of both parenchymal (hepatocytes) and immune cells (macrophages, dendritic cells, T-cells, NK/NKT-cells), where hepatocytes represent the majority of the cell populations. The role of inflammasomes has been mostly studied in immune cells, but there is increasing evidence that NLRs exist in non-immune cells as well, including keratinocytes (159, 166), myoblasts (167), fibroblasts and endothelial cells (168), osteoblasts (169), spinal cord motoneurons (170), pyramidal neurons and oligodendrocytes (159).

The liver resident macrophages, Kupffer cells produce significant amount of IL-1β that would suggest (171) the presence of inflammasomes, however, surprisingly, *Kummer et al.* showed that certain macrophages, including Kupffer cells are negative for NLRP1 staining (159). The presence of NLRP3 inflammasome and/or inflammasome activation has been also shown in sinusoidal endothelial cells (172) and stellate cells (173). However, to our best knowledge, there are no published data on hepatocytes.

The NLRs are just one components of the inflammasome complex, most of the inflammasome require an adaptor protein ASC, and the expression of caspase-1 is also prerequisite of the inflammasome assembly. ASC is expressed in several tissues, including hepatocytes and interlobular bile ducts (174), stellate cells (173). A marked, constitutive expression of caspase-1 (ICE) has been reported in the liver (175). The cell-specific expression of the inflammasome in the liver is shown as *Figure 11*.

Of course, the presence of inflammasome components is required but does not necessarily mean the activation of the complex. We will discuss below the relevance of inflammasomes in the different liver diseases.

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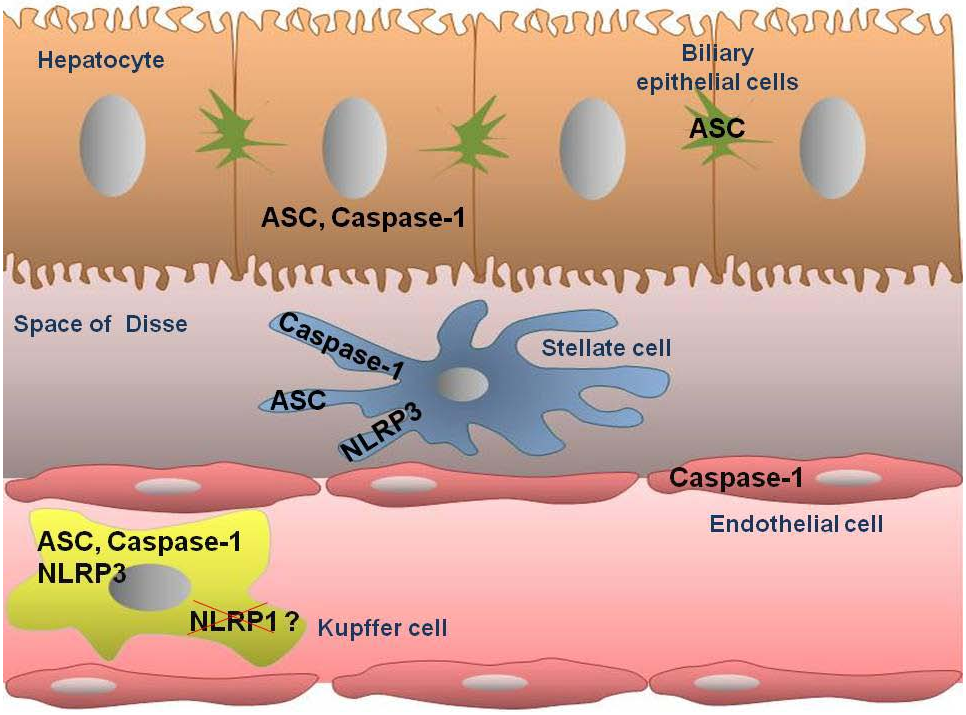

Figure 11. *Expression of inflammasome components in the liver*

2.2.3.6. Role of inflammasomes in liver diseases

The role of inflammasome activation has been implicated in several liver diseases including acetaminophen-induced liver injury (176), ischaemia-reperfusion liver injury (177), *P.acnes* plus endotoxin-induced liver injury model (178) and liver fibrosis (173).

There is increasing evidence that gut microbiota, increased gut permeability and endotoxin play a crucial role in the pathogenesis of both alcoholic (ASH) and non-alcoholic steatohepatitis (NASH) (60). Therefore, our aim was to explore the role of inflammasomes in NASH.

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3. AIM OF THE THESIS

The aim of thesis was to explore the role of innate immunity in the pathogenesis of NASH. The first part of the work focus on the role of the Gram negative bacterial wall component endotoxin and its receptor, Toll-like receptor 4 in the development of diet-induced steatohepatitis and fibrosis. The second part of the work was designed to investigate the role of the pro-inflammatory cytokine IL-1β and the inflammasome complexes that are responsible for the IL-1β-maturation in the pathogenesis of NASH. Finally, with the third part of the work we aimed to explore the pathogenesis behind the susceptibility of fatty liver to viral diseases.

3.1. To examine the role of toll-like receptor 4 signaling in the pathogenesis of NASH

There is increasing evidence that non-alcoholic steatohepatitis is accompanied with increased gut permeability and increased serum endotoxin levels (80-85). Furthermore, our group previously showed increased susceptibility to gut-derived endotoxin, lipopolysaccharide (LPS) in steatohepatitis (44). Toll-like receptor 4 (TLR4) and MD2 are the major receptors for LPS (99). Therefore the aims of the present study were:

- To investigate the role of TLR4 and its adaptor MD2 in the development of diet-induced hepatic fat accumulation, liver injury, inflammation and fibrosis. To perform the experiments we employed wild type, TLR4- and MD2-deficient mice fed with methinone-choline deficient (MCD) diet to induce steatohepatitis.

3.2. To examine the role of IL-1β and inflammasomes in the pathogenesis of NASH

The intracellular multiprotein complexes called inflammasomes are responsible for the maturation of the pro-inflammatory cytokine IL-1β that plays important role in numerous chronic and acute inflammatory diseases. Bacterial endotoxin, that has been implicated as 2nd hit in the NASH pathogenesis, is a key factor of the inflammasome activation. Therefore we aimed to investigate the role of the inflammasomes and IL-1β in the pathogenesis of NASH. The aims in details were:

- To test whether there is inflammasome activation and increased IL-1β production in animal models of liver steatosis (ob/ob mice and short term high fat diet feeding) and steatohepatitis (MCD diet-induced steatohepatitis and long term high fat diet feeding).
- To test whether hepatocytes express the inflammasomes.
- To explore potential inflammasome activators in steatohepatitis performing in vitro experiments on immune cells (RAW macrophages and isolated murine liver mononuclear cells) and hepatocytes (Hepa 1-6 cells and primary murine hepatocytes).
- To investigate the clinical significance of the inflammasomes and IL-1 signaling in the development of MCD diet-induced steatohepatitis using mice deficient in the following genes: 1. ASC (inflammasome adaptor), 2. Caspase-1 (inflammasome effector), 3. IL-1 receptor.
- To check the inflammasome expression in liver biopsy samples from NASH patients.

3.3. To examine the pathomechanism of decreased antiviral response in steatohepatitis

As we mentioned above, the co-morbidity of NASH with RNA viral infections, such as hepatitis C and HIV virus remains a clinical challenge and the susceptibility of fatty liver to virus-induced liver damage urges the better understanding of changes of antiviral immune responses in steatotic livers. Therefore the aims of the present study were:

- To test the hypothesis that mice with steatohepatitis are more susceptible to virus induced liver injury and if yes to explore the underlying pathomechanism. To perform the experiment we employed mice fed with MCD diet to induce steatohepatitis and challenged them with Poly I:C to mimic viral infection. We evaluated the liver injury using biochemical and histological methods.
- To investigate whether mice with steatohepatitis have impaired antiviral immunity to viral challenge and explore the underlying pathomechanism. To perform the experiment we used the MCD-diet induced animal model of steatohepatitis, challenged the mice with Poly I:C as a mimic of viral infection and evaluated the mounted antiviral response including interferon and cytokine production. In addition we investigated the intracellular signaling cascade step by step induced by Poly I:C.

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4. MATERIALS AND METHODS

4.1 Animal studies

This study was approved by Institutional Animal Use and Care Committee (IACUC) at University of Massachusetts (UMASS) Medical School.

Six-eight week-old C57Bl/6 wild type (wt) mice (n=6-16/group) were fed with either methionine-choline deficient (MCD) diet for 5 or 8 weeks; or high fat diet (HFD; *Harlan Laboratories Inc., South Easton, MA, USA*) for 4 weeks or 9 months. Control mice received either an MCD-identical, but DL-methionine (3 g/kg) and choline bitartrate (2 g/kg) supplemented (MCS) diet *(Dyets Inc., Bethlehem, PA, USA),* or regular rodent chow diet. We also used 9 weeks old, female leptin deficient (ob/ob; B6.V-Lep ob/J from Jackson Laboratories) mice with their own age and gender-matched control group (C57Bl/6J). All mice had unrestricted access to water. The presence of steatohepatitis was proven histologically in the MCD diet-fed mice, while fat deposition was proven by liver triglyceride assay in the HF diet fed mice and the ob/ob mice.

TLR4 ligand lipopolysaccharide (LPS) (*Sigma, St. Louis, MO, USA;* 0.5mg/bwkg to MCS/MCD mice; 12ug/mouse to ob/ob mice) or TLR3/RLR ligand polyinosinic:polycytidylic acid (Poly I:C) *(InvivoGen, San Diego, CA, USA),* a synthetic double stranded RNA (5mg/bwkg); or TLR9 ligand CpG-ODN *(InvivoGen, San Diego, CA, USA),* (5mg/bwkg) were injected intaperitoneally for 2 or 6 hours.

The following knock-out mice were used: MD-2, TLR4-, MyD88-, ASC-, caspase-1or IL-1R-defcient mice with their appropriate controls. Furthermore wild type (WT) mice transplanted with MyD88-deficient bone marrow (WT/MyD88) and MyD88-deficient mice transplanted with WT bone marrow (MyD88/WT) were employed.

4.2 Biochemical analysis and cytokine measurements

Serum alanine aminotransferase (ALT) was determined using a kinetic method *(D-TEK, Bensalem, PA, USA),* liver triglyceride levels were assessed using L-Type Triglyceride H kit *(Wako Chemicals USA Inc., VA, USA).* Serum TNFα, IL-6 and IL-1β

levels were determined by BD^{TM} Cytometric Bead Array *(BD Biosciences, Sparks, MD, USA)*, serum IFNβ and HMGB1 protein levels were measured by ELISA *(PBL Biomedical Laboratories, Piscataway, NJ, USa and IBL Transatlantic, Toronto, Canada; respectively).* Liver thiobarbituric acid reactive substances (TBARS) were assayed using whole liver homogenates and Oxi-TEK TBARS assay kit *(ZeptoMetrix Corp., Buffalo, NY, USA).*

4.3 Histopatological analysis

Sections of formalin-fixed, paraffin-embedded livers were stained with: 1) hematoxylin and eosin to assess histological features of steatohepatitis, 2) picro-sirius red stain to evaluate for hepatic collagen deposition. OilRed O tissue staining method on OCT-embedded frozen sections was used to quantify the steatosis. Liver sections were also subject to immunohistochemical staining for macrophages with monoclonal F4/80 antibody *(Abcam, Cambridge, MA)* and α–smooth muscle actin with a monoclonal antibody against α–smooth muscle actin *(Lab Vision Corporation, Fremont, CA)* using a labeled streptavidin-biotin immunoenzymatic antigen detection system *(UltraVision Mouse Tissue Detection System Anti-Mouse-HRP/DAB, Lab Vision Corp).* Image J and Microsuite software *(Olympus Soft Imaging Solutions GmbH, Munster, Germany)* was used for image analysis at indicated magnification on 20 high-power fields.

4.4 RNA analysis

RNA was purified using the RNeasy kit *(Qiagen Sciences, Maryland, USA)* and on-column DNA digestion. cDNA was transcribed with the Reverse Transcription System *(Promega Corp., Madison, WI).* Real-time quantitative polymerase chain reaction was performed using iCycler *(Bio-Rad Laboratories Inc., Hercules, CA)*; primer sequences are shown in Table 1. All specific mRNA levels were normalized against the housekeeping gene, 18S, in the same sample.

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4.5 Protein analysis

4.5.1 Preparation of cell lysates

Whole liver lysates were extracted from frozen liver using RIPA buffer *(Boston Bioproducts, Ashland, MA, USA).* Isolation of mitochondrial and cytosolic fraction from fresh liver tissue was based on the principle of differential centrifugation using Mitochondrial Extraction kit *(Imgenex Co., San Diego, CA, USA).*

4.5.2 SDS-PAGE electrophoresis

Whole liver, cytoplasmic or mitochondrial extracts were prepared. Samples with equal amounts of protein were separated in polyacrylamide gel, and proteins of interest were identified on the nitrocellulose membrane with specific primary antibodies followed by horseradish peroxidase–labeled secondary antibodies and chemiluminescence assay. The following antibodies were employed: MAVS *(Santa Cruz Biotechnology Inc.; Cell Signaling)*, cytochrome c *(Imgenex)*, caspase-1 p10 *(Santa Cruz Biotechnology Inc.)*, cleaved caspase-8 *(Imgenex)*, RIP3 *(Abcam)*, PSMA7 *(Abcam), HMGB1 (Abcam),* phoshoserine *(Abcam)*, IRF3 *(Cell Signaling)*, phosphoIRF3 *(Cell Signaling)*, IL-1β *(R&D)*, β-actin *(Abcam)*, β-tubulin *(Abcam),* Tim23 *(BD Biosciences).*

4.5.3 Native gel electrophoresis

Native PAGE Novex Bis-Tris Gel System *(Invitrogen Life Science, Carlsbad, CA, USA)* was used. Liver samples were lysed using 5% Digitonin as mild detergent and separated on Native PAGE Novex 3-12% Bis-Tris Gels. Proteins were transferred to PVDF membrane, fixed with 8% acetic acid diluted in distilled water and identified with specific primary antibodies followed by HRP labeled secondary antibodies and chemiluminescence assay.

4.5.4 Immunoprecipitation

Whole liver lysates were precleared with anti-rabbit IgG beads followed by overnight incubation with 5ug of the primary antibody (PSMA7 or MAVS) and precipitated with IgG beads. The immunprecipitates were lysed and denatured using β-

mercaptoethanol containing buffer and heating. The proteins were separated on polyacrylamid gel, transferred to nitrocellulose membrane and detected by specific antibodies (MAVS, PSMA7).

4.6 Functional assays

4.6.1 Caspase-activity assays

Caspase-1 activity was determined in freshly prepared whole liver lysates using colorimetric assay. Caspase-1 activity analysis is based on the cleavage of substrate WEHD-pNA *(R&DSystems, Minneapolis, MN, USA).*

Caspase-3 activity was determined in freshly prepared whole liver lysates using colorimetric assay. Caspase-3 activity analysis is based on the cleavage of substrate DECD-pNA *(GenScript, Piscataway, NJ, USA).*

4.6.2 NADPH activity assay

NADP+/NADPH concentrations from whole liver extracts with comparable protein amounts were determined using EnzyChrom NADP+/NADPH assay kit (ECNP-100) *(BioAssay Systems, Hayward, CA)*, as manufacturer recommended.

4.6.3 Cytotoxicity assay

The lactate dehydrogenase (LDH) assay *(Sigma-Aldrich, St. Louis, MO, USA)* was used to measure the amount of cytoplasmic LDH released into the medium as an indicator of membrane integrity and cell viability.

4.7 In vitro experiments

Cell lines or primary cells were stimulated with LPS (100 or 1000ng/ml for 2, 6, 18hours), fatty acids (palmitic acid with BSA 0.33mM, 0.165mM; oleic acid 0.66mM, 0.33mM; linoleic acid 0.66mM, 0.33mM for 2,6,18,24,36 hours) or their combinations with or without ZVAD (40μM). Poly I:C (10μg/ml) was used to stimulate hepatocytes with or without Lipofectamin 2000 (6 hours.)

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4.7.1 Cell lines

Hepa1-6 mouse hepatoma cell-line and RAW 264.7 mouse leukemic monocyte-macrophage cell-line were employed.

4.7.2 Primary cells

Animals received anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg); the livers were perfused with Hank's balanced saline solution (HBSS) followed by *in vivo* digestion with 0.33 mg/ml Liberase RI Enzyme *(F. Hoffmann-La Roche Ltd; Basel Switzerland)* in HBSS. The LMNCs and hepatocytes were purified from whole liver cell suspension obtained after tissue disruption using centrifugation at slow speed (500*g*). Hepatocytes were washed twice with 2% fetal bovine serum (FBS) containing PBS and were plated on collagen-coated plates. The LMNCs were further purified by subsequent isolation in Percoll 40/70 gradient density at 800*g* and harvested from the gradient interface. Purity of cell population was assessed by qPCR.

4.8 Flow cytometry analysis

Liver mononuclear cells were washed in saline supplemented with 2% fetal bovine serum (FBS) and stained for surface NK cell marker NK1.1 *(BD Bioscience, San Jose, CA).* In some experiments LMNCs were stimulated with a cocktail of PMA (50 ng/ml), ionomycin (1µg/ml), and brefeldin A (10µg/ml) in RPMI1640+10% FBS for 4 hours and stained for CD68 and intracellular TNFα using specific fluorescent labeled antibodies and CytoFix/CytoPerm Kit *(BD Bioscience, San Jose, CA).* The cells were gated by size and granularity and their fluorescence was analyzed using the LSR flow cytometer.

4.9 Human liver samples

The study meets the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Committee for the Protection of Human Subjects in Research at the University of Massachusetts. All participants gave a written consented to participate in the study.

Human liver tissue was obtained from biopsies from six (2 males and 4 females; age: 45±8 years), clinically and biopsy-proven NASH patients. The histology showed steatosis (<1/3 hepatocytes: n=2, 1-2/3 hepatocytes: n=3, >2/3 hepatocytes: n=1) with rare hepatocyte ballooning (none: n=2, <1/3 hepatocytes: n=4) and inflammation with inflammatory score 1-4. Lobular inflammation was present in 5 patients. Fibrosis was not detected in any of the patients. Human liver tissue from chronic hepatitis C infected patients (n=5) were used as diseased controls. Human normal liver (n=4) total RNA was purchased from *OriGene Technologies (Rockville, MD, USA)*

4.10 Statistical analysis

Statistical significance was determined using the nonparametric Kruskal-Wallis test, Mann-Whitney tests, where appropriate. Data are shown as mean ± standard error and were considered statistically significant at $p \leq 0.05$.

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5. RESULTS

5.1 Deficiency in myeloid differentiation factor-2 (MD2) and toll-like receptor 4 (TLR4) expression attenuates non-alcoholic steatohepatitis and fibrosis in mice

5.1.1 MD-2 or TLR4 protects from MCD diet-induced liver fat deposition and inflammation

MD-2 and TLR4 complex is the major receptor for endotoxin (179) that has been shown to contribute to activation of the inflammatory cascade in alcoholic steatohepatitis (ASH) leading to liver damage. Given the common pathophysiological features of ASH and NASH, we aimed to identify the role of MD-2/ TLR4 complex in an experimental model of NASH using mice deficient in MD-2 or TLR4 and their genotype control counterparts. Feeding a methionine-choline-sufficient (MCS) diet resulted in no signs of hepatic steatosis or inflammation in any of the mice *(Figure 12-16)*. In contrast, mice of control genotypes fed a methionine-choline-deficient (MCD) diet for 8 weeks developed significant hepatic steatosis; MD-2- and TLR4-deficient mice on MCD diet showed lower liver fat accumulation, identified after OilRed O staining, compared to the mice of control genotypes *(Figure 12)*. Consistent with the development of hepatic steatosis, liver triglyceride levels were significantly increased in MCD-diet-fed control genotype mice but to a significantly lower extent in MD-2- or TLR4-deficient mice *(Figure 13)*. These findings suggested that TLR4/MD2 complex deficiency is partially protective against MCD-induced liver steatosis.

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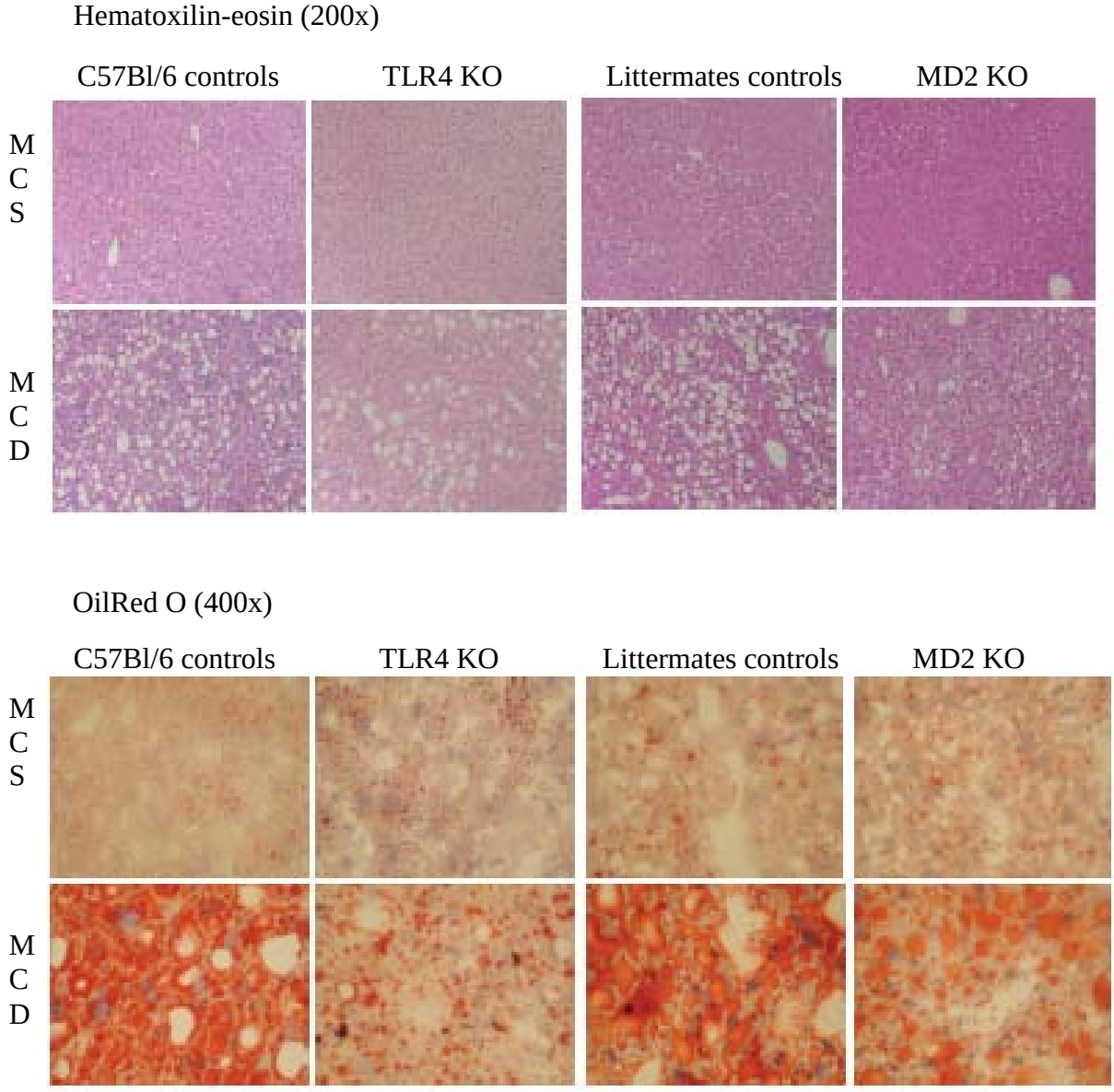

Figure 12. *Mice of control genotypes and those deficient (knock-out, KO) in TLR4 (TLR4 KO) and MD-2 (MD-2 KO) were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Liver tissue was subjected to H&E (top panel) and OilRed O (bottom panel), one representative slide from n=6-16/group is shown.*

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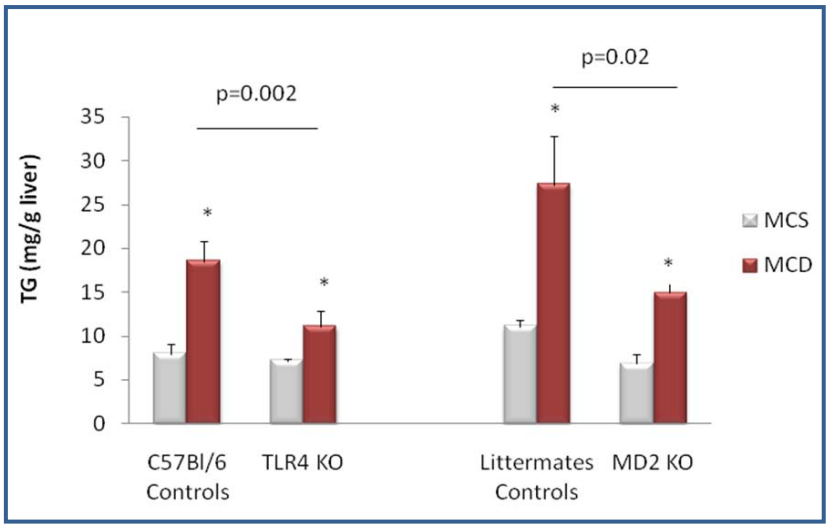

Figure 13. *Mice of control genotypes and those deficient (knock-out, KO) in TLR4 (TLR4 KO) and MD-2 (MD-2 KO) were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Liver triglycerides were determined as described in the Methods. (*) represents $p<0.05$ compared to corresponding MCS group; n=6-16/group*

Feeding of MCD diet lead to accumulation of inflammatory cells into the liver in mice of control genotypes, and to a lesser extent in MD-2- or TLR4-KO mice, as indicated by the increase in content of F4/80+ cells in the livers of MCD-fed animals, compared to MCS diet-fed controls *(Figure 14).*

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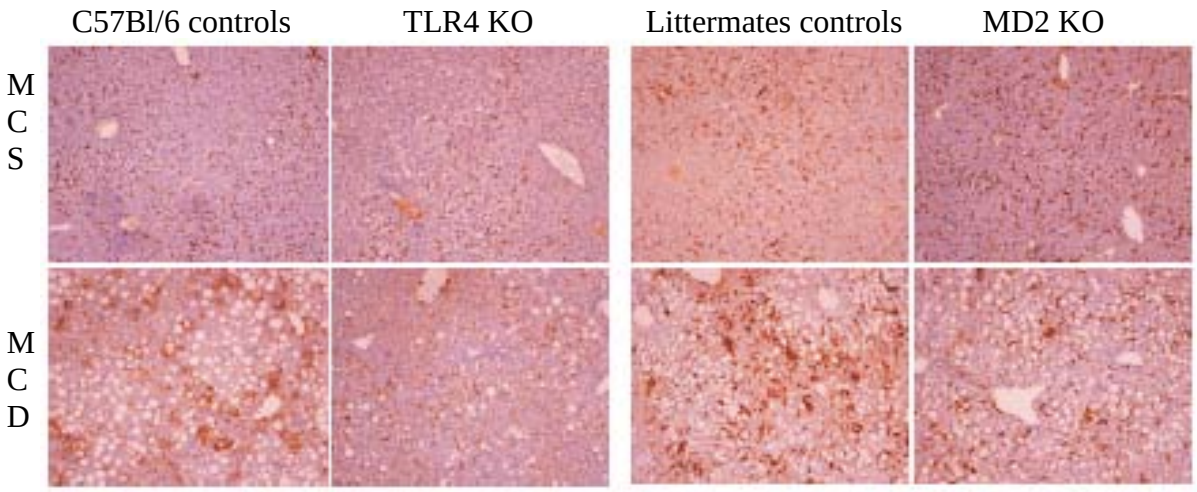

Figure 14. *Mice of control genotypes and those deficient (knock-out, KO) in TLR4 (TLR4 KO) and MD-2 (MD-2 KO) were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Liver tissue was subjected to F4/80 immunohistochemistry, one representative slide from n=6-16/group is shown.*

Further, the proportion of TNFα-producing CD68+ macrophages was increased in MCD-fed compared to MCS-fed genotype controls (Figure 14). More importantly, TLR4 deficiency protected from MCD diet-induced accumulation of the TNFα-producing CD68+ macrophages in the liver *(Figure 15).*

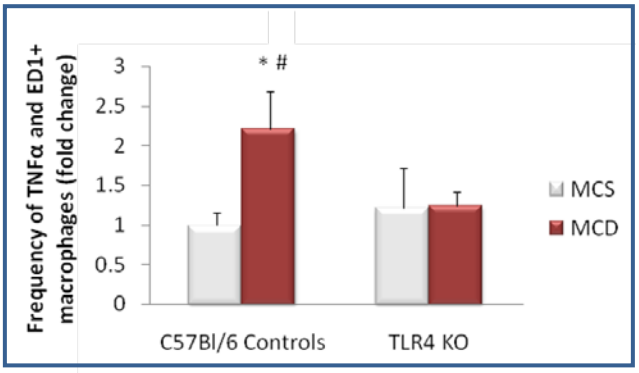

Figure 15. *Mice of control genotypes and those deficient (knock-out, KO) in TLR4 (TLR4 KO) and MD-2 (MD-2 KO) were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Liver macrophages were isolated and stained*

for TNFα and the macrophage marker CD68 (ED-1) after cell permeabilization; FACS analysis of changes in frequency of TNFa/CD68 double-positive cells compared to MCS-fed genotype control is shown. (*) represents p<0.05 compared to corresponding MCS group; (#) p<0.05 compared to TLR4 KO MCD group; n=6-16/group

A significant increase in serum alanine aminotransferase (ALT), suggesting on-going liver damage, was observed in the MCD-diet-fed control genotype mice, and this correlated well with the steatohepatitis; however, the ALT increase was significantly attenuated in MD-2- and TLR4-deficient mice *(Figure 16).*

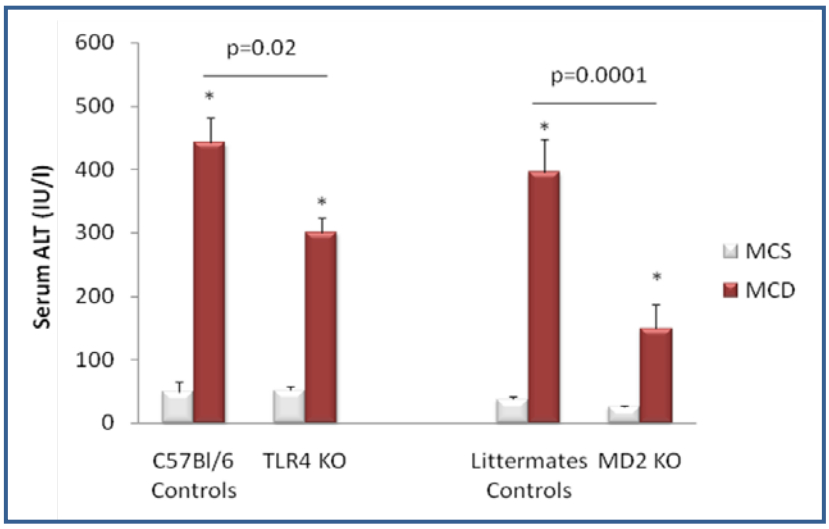

Figure 16. *Mice of control genotypes and those deficient (knock-out, KO) in TLR4 (TLR4 KO) and MD-2 (MD-2 KO) were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Serum alanine aminotransferase (ALT) values were determined as described in the Methods. (*) represents p<0.05 compared to corresponding MCS group; n=6-16/group*

TNFα has been suggested as a central pro-inflammatory cytokine that is produced by activated inflammatory cells and mediates insulin resistance and hepatocyte apoptosis in liver disease (180,181). Consistent with activation of the inflammatory cascade, serum TNFα level was increased in MCD-diet-fed control genotype mice compared to the MCS-

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diet-fed controls *(Figure 17)*. In contrast, MCD-induced TNFα was significantly lower in MD-2- or TLR4-deficient MCD diet-fed mice *(Figure 17)*. These data suggested that TLR4/MD2 complex deficiency is partially protective against MCD-induced liver inflammation and damage.

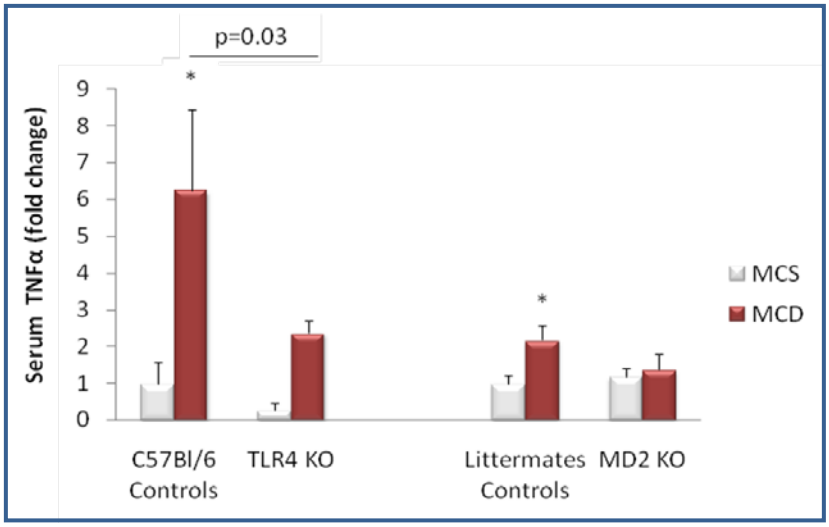

Figure 17. *Mice of control genotypes and those deficient (knock-out, KO) in TLR4 (TLR4 KO) and MD-2 (MD-2 KO) were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Serum TNFα level was determined using the Multiplex assay. (*) represents p<0.05 compared to corresponding MCS group; n=6-16/group*

5.1.2 MD-2 and TLR4 deficiency attenuates oxidative stress

Increased lipid peroxidation and oxidative stress are key in development of steatosis in non-alcoholic fatty liver disease (182). We identified significantly higher levels of liver thiobarbituric acid substances (TBARS), indicative of lipid peroxidation, in MCD-diet compared to the MCS diet-fed genotype control mice *(Figure 18)*. Consistent with our hypothesis that MD2/TLR4 complex plays a role in NASH, we found significantly reduced induction of TBARS in the livers of MCD-diet-fed MD-2- and TLR4-deficient mice *(Figure 18)*.

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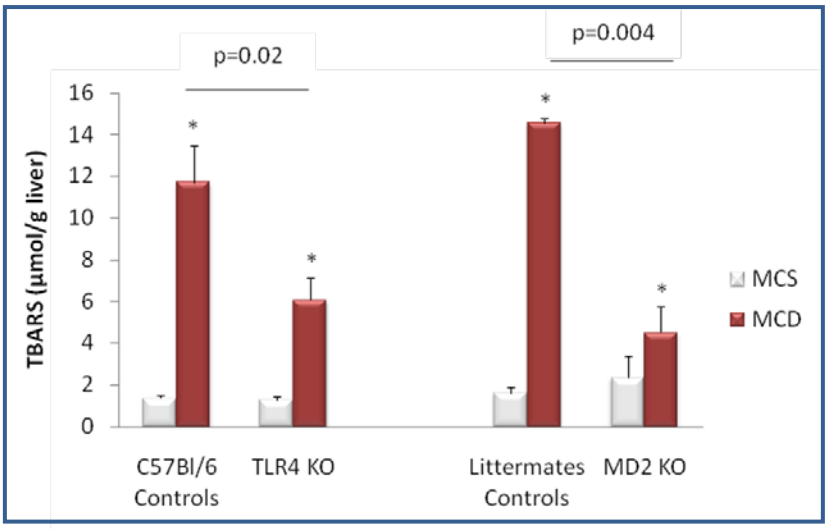

Figure 18. *Mice of genotype control, TLR4 KO, and MD-2 KO were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Liver TBARS levels were analyzed as described in Methods. (*) represents $p<0.05$ compared to corresponding MCS group; n=6-16/group*

NADPH oxidases play an important role in the generation of reactive oxygen radicals (183,184). The classic NADPH complex is composed of at least six components, which include two trans-membrane flavocytochrome b components (gp91phox and p22phox) and four cytosolic components (p47phox, p67phox, p40phox and Rac-1 protein) (184). TLR4-mediated signals are strong inducers of NADPH transcription and functional activity (183). Investigation of NADPH oxidase expression revealed a significant upregulation of the cytoplasmic components of the NADPH oxidase, including p47phox *(Figure 19A)* and p67phox *(Figure 19B)*, in MCD-diet-fed animals of control genotypes. The membrane-associated components of the NADPH complex, gp91phox *(Figure 19C)* and p22phox *(Figure 19D)*, were also up-regulated at the mRNA level in the livers of MCD-diet-compared to the MCS diet-fed mice of control genotypes. Deficiency in MD-2 or TLR4 abrogated the MCD-induced up-regulation of all of the NADPH oxidase subunits *(Figure 18A-D)*, suggesting that NADPH-mediated oxidative stress is dependent on MD-2 and TLR4 expression in this model.

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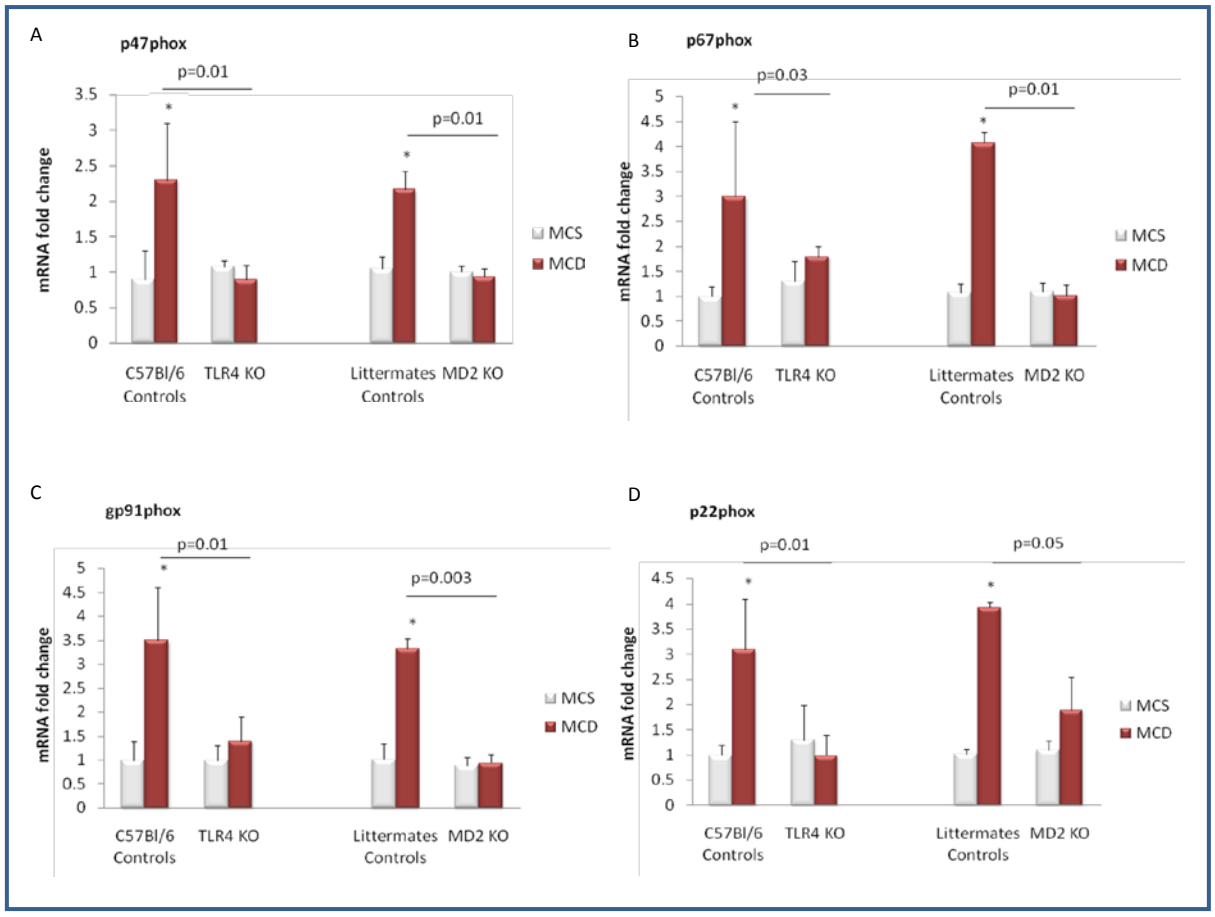

Figure 19. *Mice of genotype control, TLR4 KO, and MD-2 KO were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. Expression of liver p47phox (B), p67phox (C), gp91phox (D), and p22phox (E) were quantified by qPCR using specific primers and normalization against housekeeping gene, 18S. (*) represents p<0.05 compared to corresponding MCS group; n=6-16/group*

To test for the biological significance of the mRNA increase in the NADPH subunits, we evaluated the NADPH oxidase activity. Consistent with the increased mRNA levels of NAPDH oxidase complex components, NADPH oxidase activity was elevated, as suggested by increased NADP+/NADPH ratio in livers of MCD-fed compared to MCS-fed mice of control genotypes *(Figure 20).* More importantly, we identified that both TLR4-KO and MD2-KO mice were protected from the MCD diet-induced activation of NADPH

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oxidase *(Figure 20)*. Collectively, these results indicated that MD-2/TLR4 complex–induced signals contribute to liver pathology via NADPH-dependent lipid peroxidation and oxidative stress in the MCD-diet-induced NASH model.

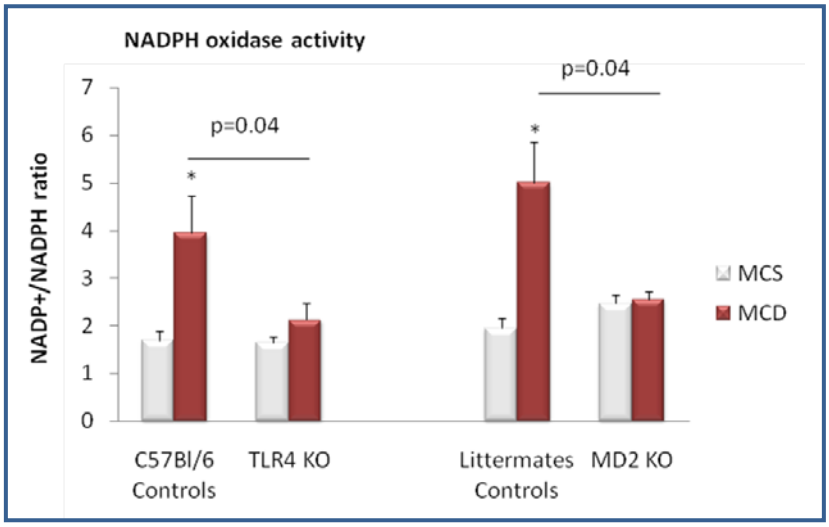

Figure 20. *Mice of genotype control, TLR4 KO, and MD-2 KO were fed methionine-choline-deficient (MCD) or methionine-choline-sufficient (MCS) diets for 8 weeks. NADPH oxidase activity was determined by measuring NADP+/NADPH ratios, was performed as described in Methods (*) represents p<0.05 compared to corresponding MCS group; n=6-16/group*

5.1.3 MD-2 and TLR4 deficiency protects from NASH-associated liver fibrosis

A key clinical challenge in human NASH is its progression to fibrosis and cirrhosis (185). In contrast to livers of MCS-diet-fed control genotype animals, Sirius red *(Figure 21,22)* and alpha smooth muscle actin (aSMA) immunohistochemistry *(Figure 23)* staining revealed that administration of MCD diet resulted in signs of fibrosis *(Figure 21-23)*. On the contrary, we found no substantial Sirius red *(Figure 21,22)* or aSMA staining *(Figure 23)* in either MD-2- or TLR4-deficient MCD-diet-fed mice. Genes associated with fibrosis, including aSMA, procollagen-1 and TGFβ *(Figure 24A,B,C, respectively)*, were significantly upregulated at the RNA level in MCD-diet-fed control genotypes, but not or less extent in MD-2 and TLR4 deficient mice.

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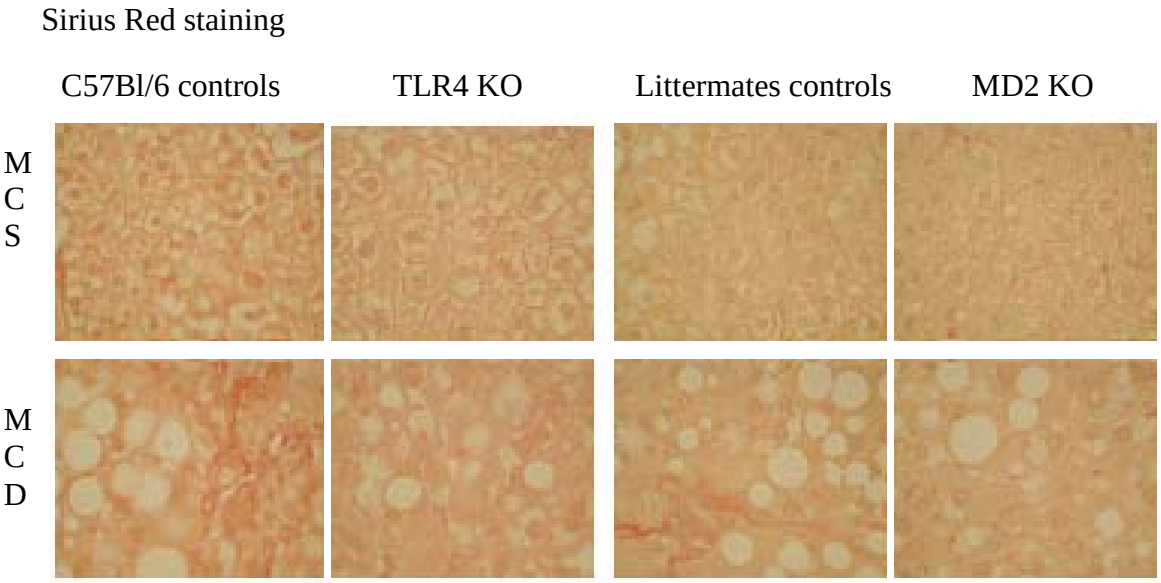

Figure 21. *The livers of MCD- and MCS-diet-fed genotype controls, MD-2 KO and TLR4 KO mice were stained with Sirius red; shown here are representative pictures from n=6-16/group.*

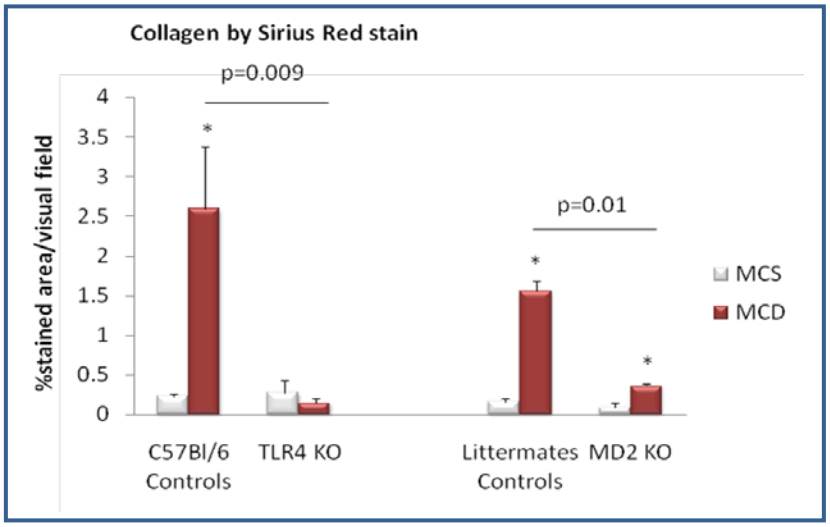

Figure 22. *The livers of MCD- and MCS-diet-fed genotype controls, MD-2 KO and TLR4 KO mice were stained with Sirius red. Sirius red positive areas were quantified using*

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Image J software (B). (*) represents p<0.05 compared to corresponding MCS group; n=6-16/group

Alpha smooth muscle actin immunohistochemistry

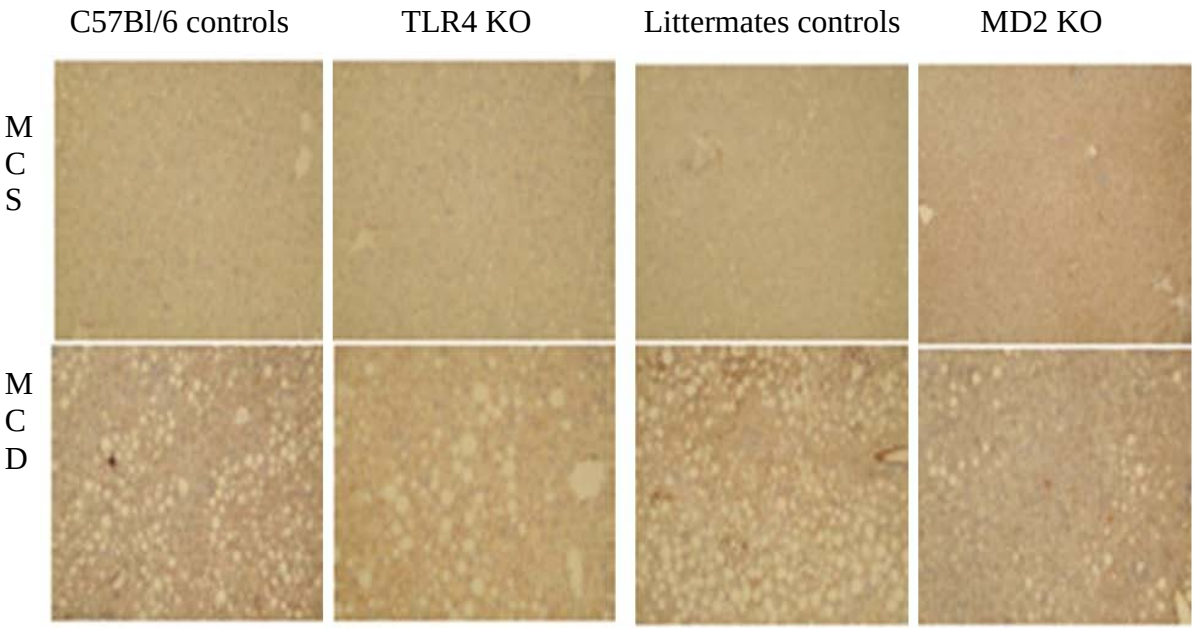
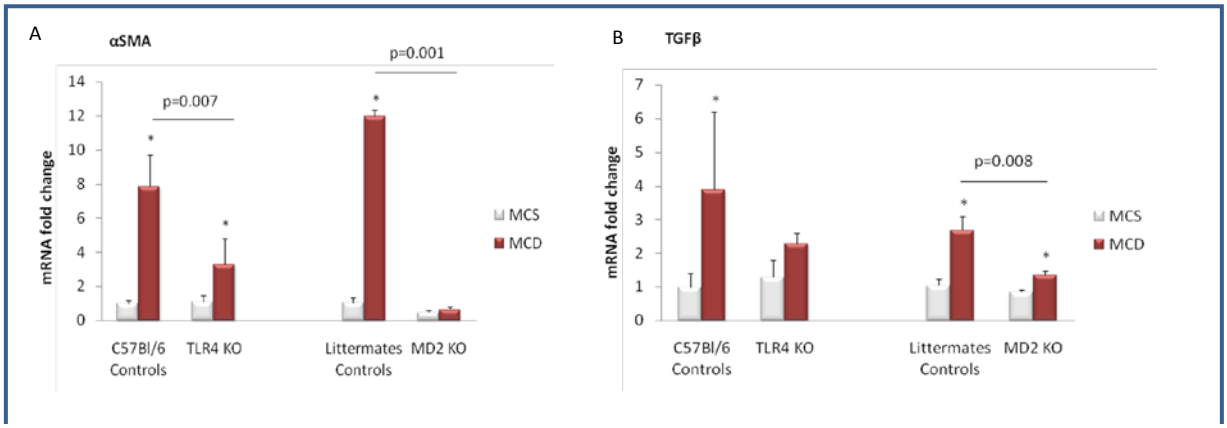


Figure 23. *The livers of MCD- and MCS-diet-fed genotype controls, MD-2 KO and TLR4 KO mice were stained with alpha smooth muscle actin (aSMA) immunohistochemistry; shown here are representative pictures from n=6-16/group.*



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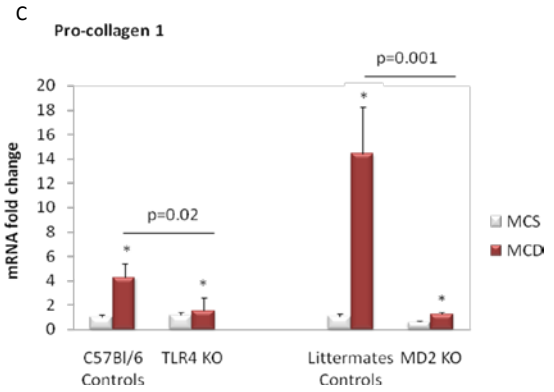

Figure 24. *Genes associated with fibrosis, including αSMA (A), procollagen-1 (B) and TGFβ (C) were quantified by qPCR using specific primers and normalization against housekeeping gene, 18S; data (*) represents p<0.05 compared to corresponding MCS group; n=6-16/group*

Liver fibrosis involves inflammation-driven tissue remodeling; matrix metalloproteinases (MMP) and their specific tissue inhibitors (TIMPs) closely regulate the metabolism of the extracellular matrix (186,187). The expression of matrix metalloproteinase 2 (MMP-2) *(Figure 25A)* and the tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) *(Figure 25B)* were increased in livers of MCD- compared to MCS-diet-fed mice of control genotypes; the induction of these genes was significantly attenuated in the absence of MD-2 or TLR4 expression.

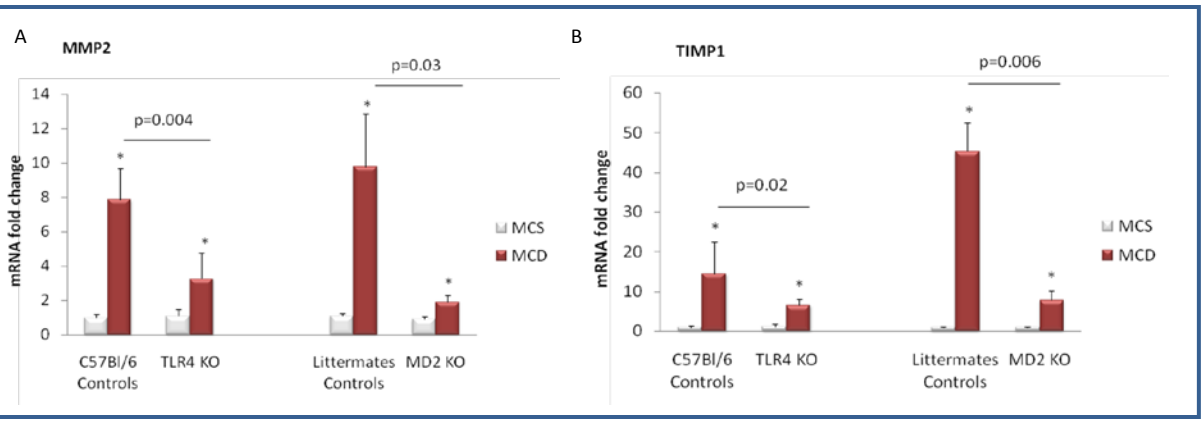

Figure 25. *The livers of MCD- and MCS-diet-fed genotype controls, MD-2 KO and TLR4 KO mice were analyzed for MMP-2 (A) and TIMP-1 (B) expression by qPCR using specific primers and normalization against housekeeping gene, 18S; (*) represents p<0.05 compared to corresponding MCS group; n=6-16/group*

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5.2 Fatty acids and endotoxin activates the inflammasome in non-alcoholic steatohepatitis

Given the role of endotoxin and TLR4, and the fact that there is crosstalk between TLR and NLR signaling, furthermore, that endotoxin is a known activator of inflammasomes, next step we aimed to investigate the role of inflammasomes in the pathogenesis of NASH.

5.2.1 MCD diet-induced steatohepatitis is associated with increased IL-1β production and inflammasome activation in the liver

The MCD diet model of non-alcoholic steatohepatitis (NASH) is characterized by steatosis and prominent inflammation indicated by increased inflammatory cell infiltrates in the liver and elevated serum pro-inflammatory cytokine levels (1). Here we found that among other pro-inflammatory cytokines (44) the levels of serum IL-1β *(Figure 26A)* as well as hepatic IL-1β mRNA *(Figure 26B)* were significantly increased in the livers of MCD diet-fed mice compared to MCS controls. IL-1β is cleaved from pro-IL-1ß by caspase-1 that is activated by the inflammasome complex (117). Thus, we tested expression of the inflammasome components, NALP3, pro-caspase-1 and the NALP adaptor molecule, ASC, and found that all were up-regulated at the mRNA levels in livers of mice with MCD compared to MCS diet feeding *(Figure 27).* Association of NALP3 with procaspase-1 via the adaptor molecule ASC results in auto-activation of the inflammasome complex and activation of caspase-1 that cleaves IL-1ß (117). Caspase-1 activity was significantly increased in livers of MCD diet-fed mice compared to MCS controls *(Figure 28A)*. Consistent with increased inflammasome expression and caspase-1 activation, the levels of mature IL-1β protein were increased in the liver *(Figure 28B)* of MCD diet-fed mice compared to MCS controls. In addition to inflammation, there were increased triglyceride levels in the liver of MCD diet-fed mice indicating steatosis *(Figure 29).*

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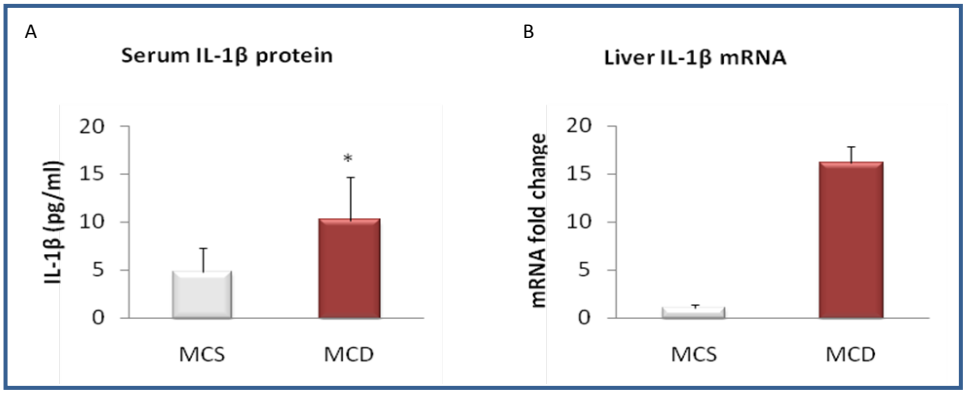

Figure 26. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks. Serum (A) and liver mature IL-1β (B) were determined. N=6 mice/group, (*) indicates p<0.05 vs. MCS*

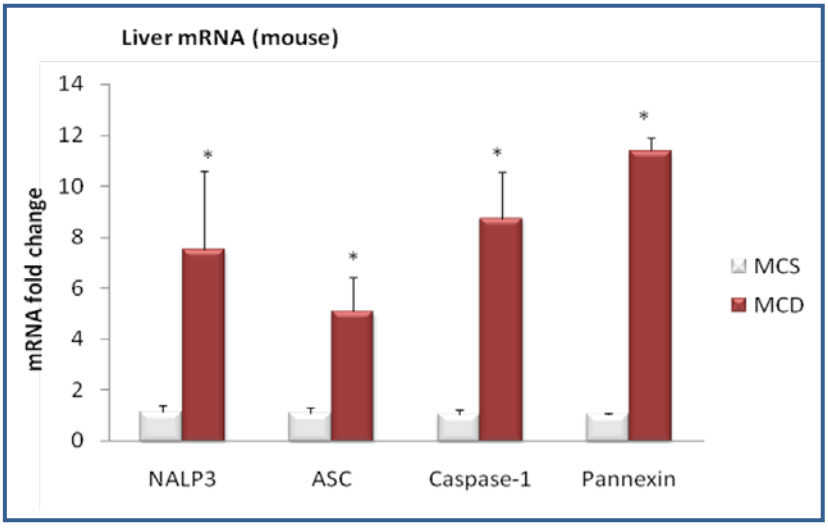

Figure 27. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks. Liver mRNA of NALP3 inflammasome complex including NALP3, pro-caspase-1, ASC, pannexin-1 was determined. N=6 mice/group, (*) indicates p<0.05 vs. MCS*

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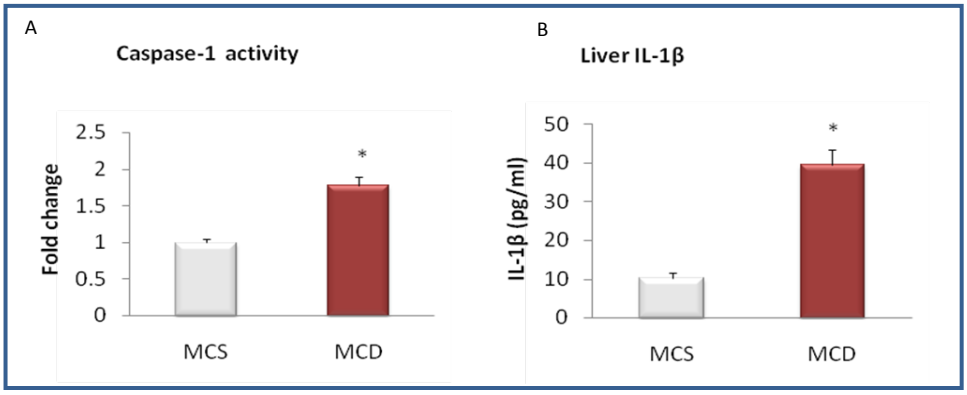

Figure 28. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks. Functional activity of the inflammasome was evaluated by measuring caspase-1 activity (A) and liver mature IL-1β protein levels (B). N=6 mice/group, (*) indicates p<0.05 vs. MCS*

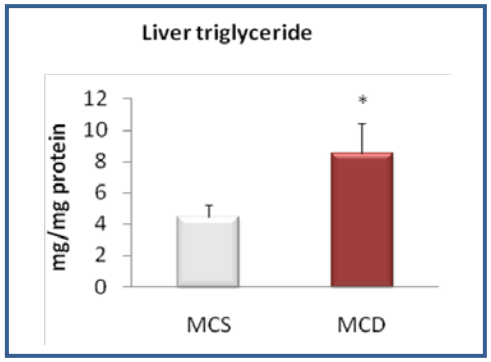

Figure 29. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks. Liver triglyceride levels were measured. N=6 mice/group, (*) indicates p<0.05 vs. MCS*

5.2.2 Long-term, but not short-term high fat diet feeding is associated with inflammasome activation in the liver

Human NAFLD includes the spectrum of fatty liver and NASH. While the MCD diet model induces NASH, high fat diet results in steatosis after 4 weeks and evidence of inflammation occurs after prolonged HFD feeding (188,189). Consistent with this, we observed an increase in liver TNFα-expression *(Figure 30)* only in livers with 9-month and not with 4-week HFD feeding.

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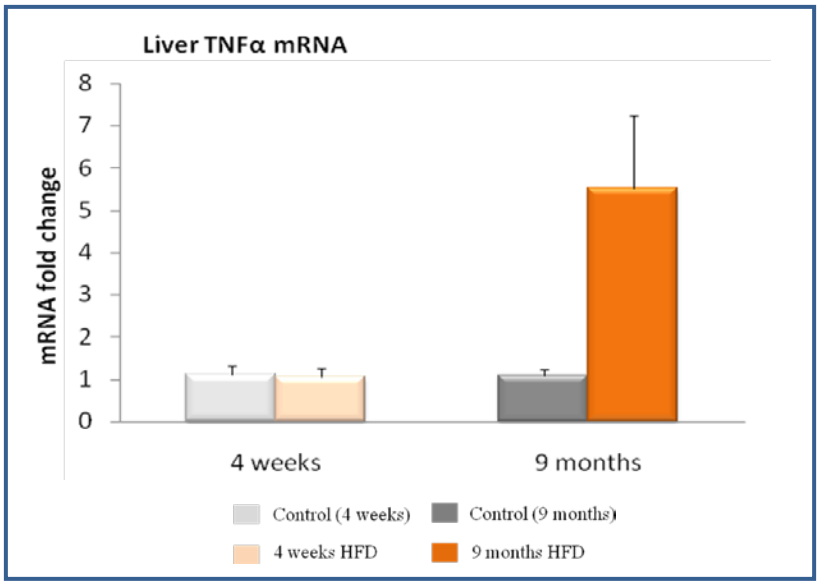
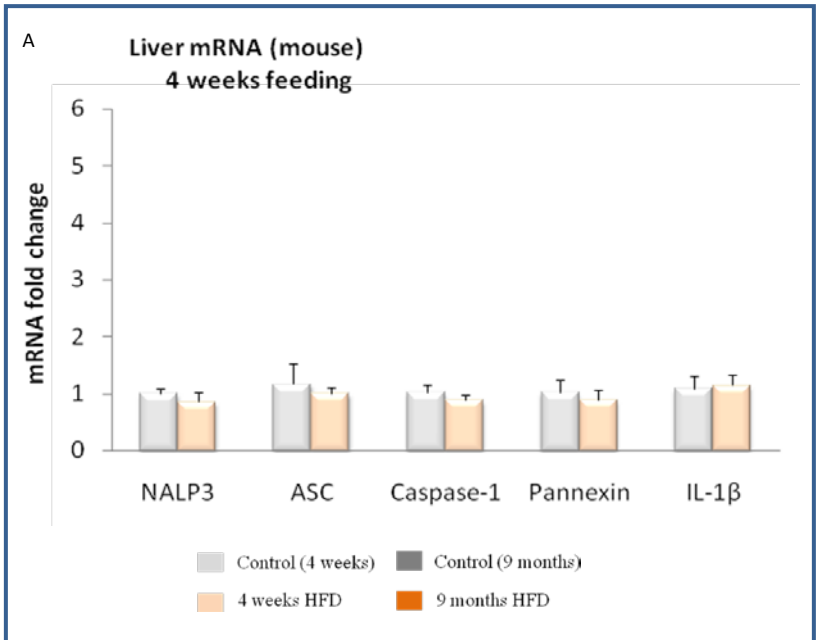


Figure 30. *C57/Bl6 mice were fed with high fat diet (HFD) or control diet for 4 weeks or 9 months. Liver mRNA of TNFα was determined by qPCR. N=6 mice/group, (*) indicates $p<0.05$ vs. corresponding control group.*

We found that 4-week HFD resulted in no increase in inflammasome expression *(Figure 31A),* while 9-month HFD induced significant up-regulation of the NALP3 inflammasome (NALP3, ASC, caspase-1, pannexin-1, IL-1β) complex at the mRNA level *(Figure 31B).*



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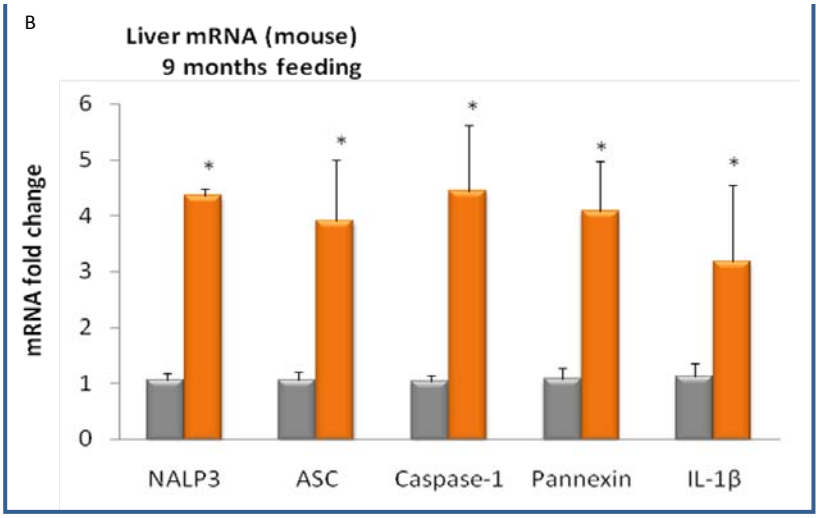

Figure 31. *C57/Bl6 mice were fed with high fat diet (HFD) or control diet for 4 weeks or 9 months. Liver mRNA of IL-1β and NALP3 inflammasome complex including NALP3, pro-caspase-1, ASC, pannexin-1 was determined after 4-week (A) and 9-month (B) HFD feeding. Functional activity of the inflammasome was evaluated by measuring caspase-1 activity (C) and liver mature IL-1β protein levels (D). Liver triglycerides were measured (E). N=6 mice/group, (*) indicates p<0.05 vs. corresponding control group.*

Inflammasome activation was indicated by increased caspase-1 activity *(Figure 32A)* and higher liver mature IL-1β protein levels *(Figure 32B)* in 9-month but not in 4-week HFD groups compared to their corresponding controls.

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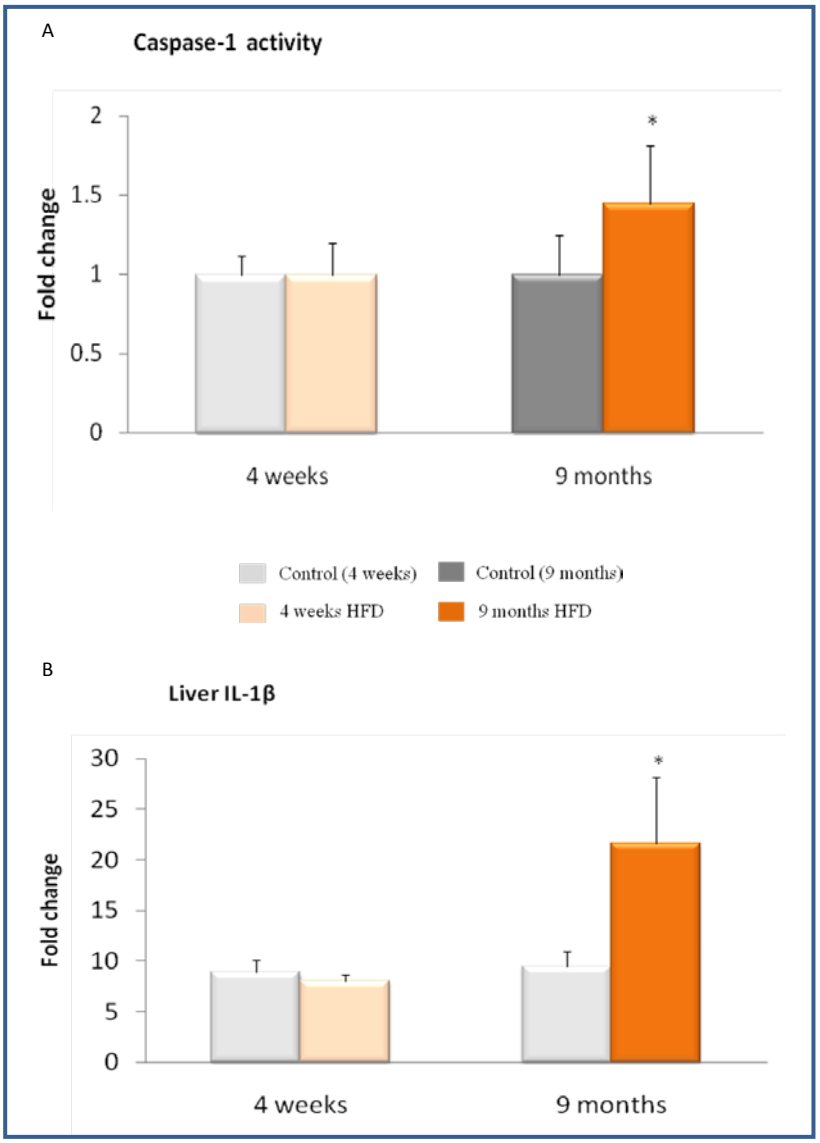

Figure 32. *C57/Bl6 mice were fed with high fat diet (HFD) or control diet for 4 weeks or 9 months. Functional activity of the inflammasome was evaluated by measuring caspase-1 activity (A) and liver mature IL-1β protein levels (B). (*) indicates $p<0.05$ vs. corresponding control group.*

Increased liver triglyceride levels indicated fat accumulation in MCD *(Figure 30)* and 9-month HFD feeding *(Figure 33)*. There was no significant difference in liver triglycerides (TG) in 4-week HFD compared to control groups *(Figure 33)*; notably this control group had significantly higher TG levels compared to other control groups.

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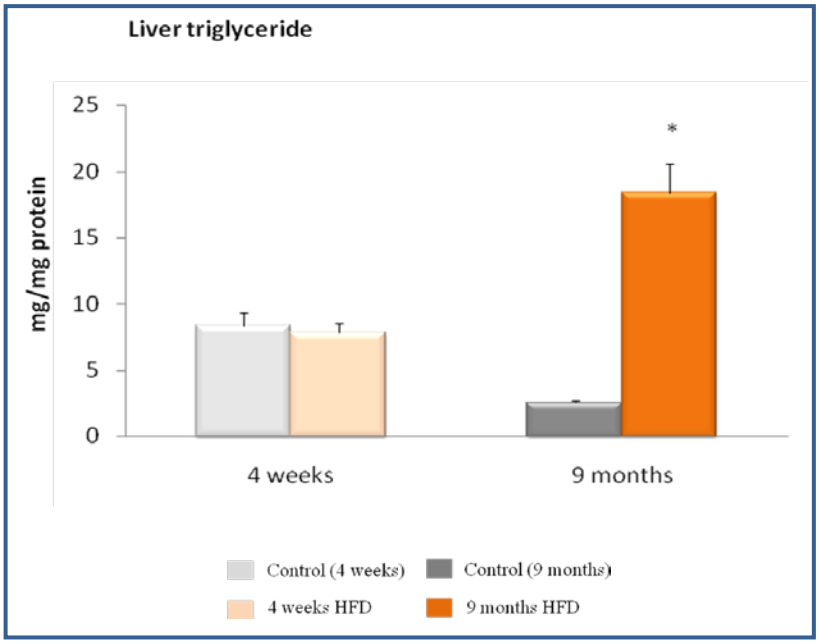

Figure 33. *C57/Bl6 mice were fed with high fat diet (HFD) or control diet for 4 weeks or 9 months. Liver triglyceride levels were measured. N=6 mice/group, (*) indicates p<0.05 vs. MCS*

Liver steatosis without features of inflammation is also prominent in leptin deficient (ob/ob) mice (190). We found no inflammasome activation in ob/ob mice compared to their controls *(Figure 34,35)* and in vivo LPS challenge failed to induce accelerated inflammasome activation in ob/ob mice compared to controls *(Figure 34,35).*

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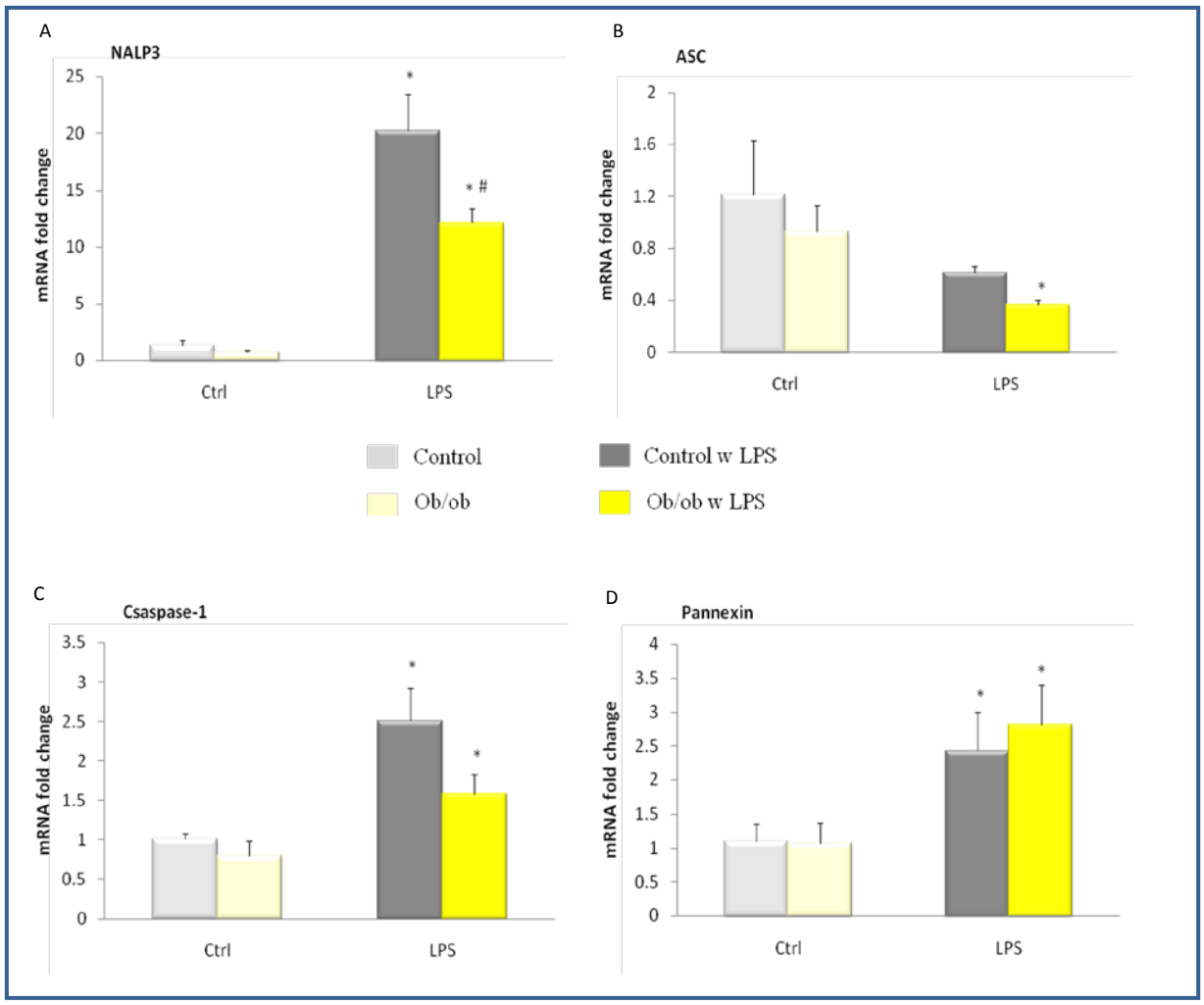

Figure 34. *Liver mRNA of NALP3 inflammasome complex including NALP3 (A), ASC (B), caspase-1 (C) and pannexin-1 (D) was determined in leptin deficient (B6.V-Lep ob/J ; ob/ob) and C57/Bl6J control mice. All the experiments were repeated after 2h LPS challenge (i.p. (A-F).) N=5 mice/group, (*) indicates p<0.05 vs. corresponding control group.*

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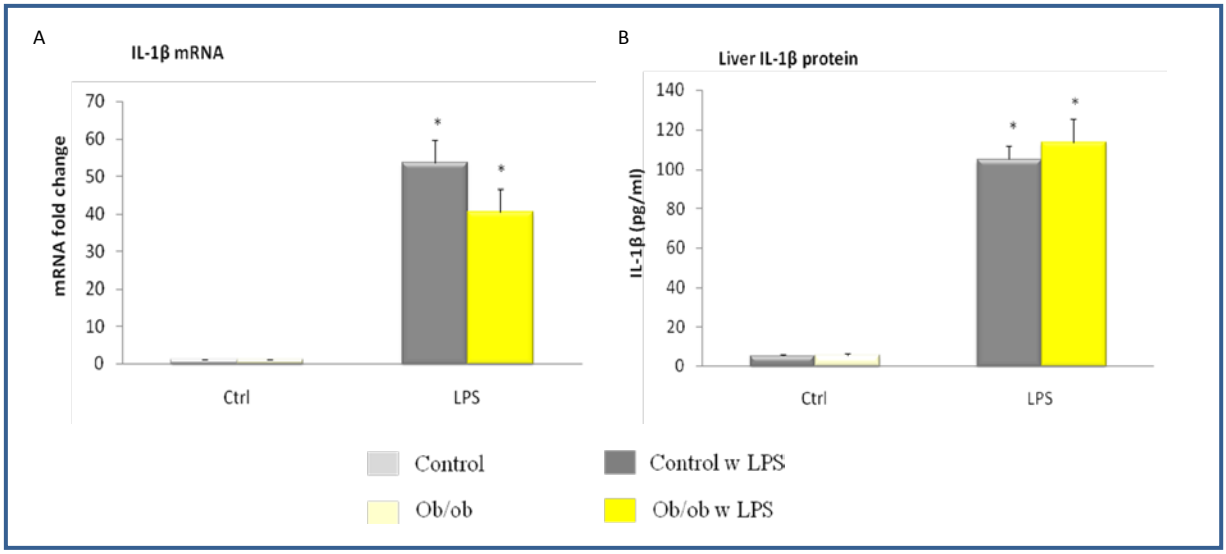

Figure 35. *Liver mRNA of IL-1β (A) was determined in leptin deficient (B6.V-Lep ob/J ; ob/ob) and C57/Bl6J control mice. Functional activity of the inflammasome was evaluated by measuring liver mature IL-1β protein levels (B). All the experiments were repeated after 2h LPS challenge (i.p. (A-F).) N=5 mice/group, (*) indicates p<0.05 vs. corresponding control group.*

5.2.3 Increased inflammasome expression in human NASH

To validate observations from the mouse models, we next evaluated human livers. There was a significant increase in inflammasome gene expression including NALP3, pro-caspase-1, ASC and pannexin-1 in livers from NASH patients compared to healthy controls *(Figure 36).* This observation in human NASH corroborated the inflammasome activation in the mouse models of NASH. Liver samples from chronic HCV infected patients also showed increased inflammasome expression, however, to a lower extent than NASH livers *(Figure 36.)*

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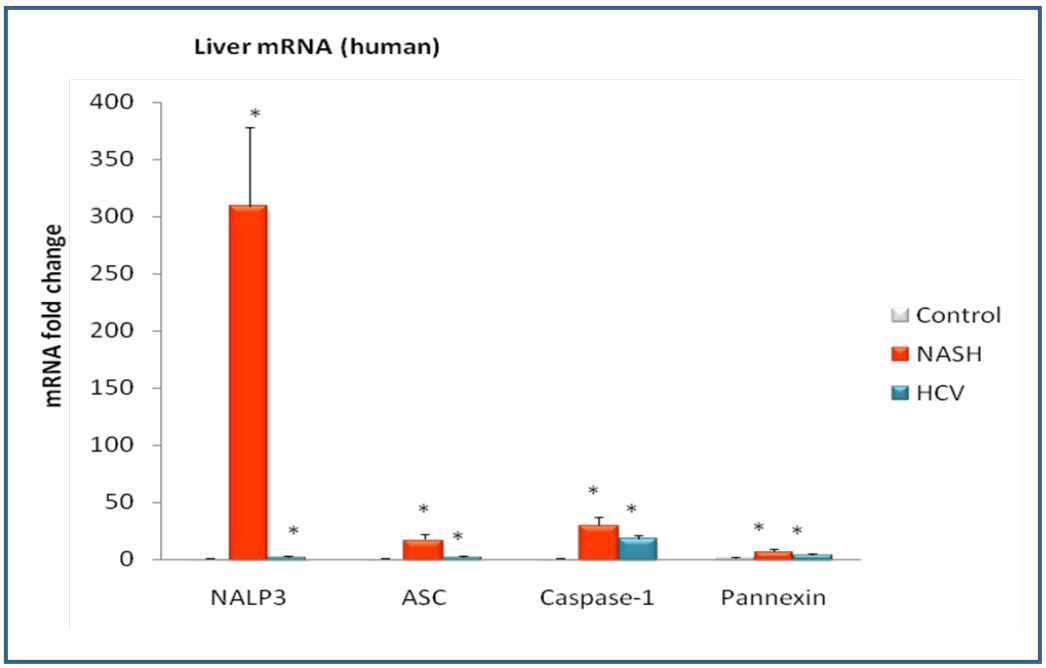

Figure 36. *The mRNA expression of NALP3, ASC, pro-caspase-1 and pannexin-1 were measured by qPCR in livers NASH patients compared to commercially available normal human liver RNA (n=4), and to liver samples from HCV-infected patients. (*) indicates $p<0.05$ vs. control.*

5.2.4 LPS induces upregulation of the inflammasome in the liver

Inflammasome activation requires two signals, usually consisting of a combination of an endogenous danger signal and a TLR ligand (117,191,192). The pathogenesis of NASH has also been linked to two "hits" (56). It has been suggested that endotoxin (LPS), presumably gut-derived, usually acts as a potent 2nd hit and aggravates liver injury (44,80,81). Here we tested whether the inflammasome could be further activated by TLR4/LPS in steatohepatitis. *In vivo* stimulation with the TLR4 ligand, LPS, lead to up-regulation of the hepatic inflammasome components NALP3, IL-1β at the mRNA level *(Figure 37A,B),* and increased IL-1β protein in the liver *(Figure 37C)* in both MCD and MCS diet-fed mice. We also noted significantly higher induction of the inflammasome in MCD compared to MCS diet-fed mice after LPS challenge. Together, these data suggested

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that NASH is associated with inflammasome activation as well as sensitization to LPS-induced upregulation inflammasome function.

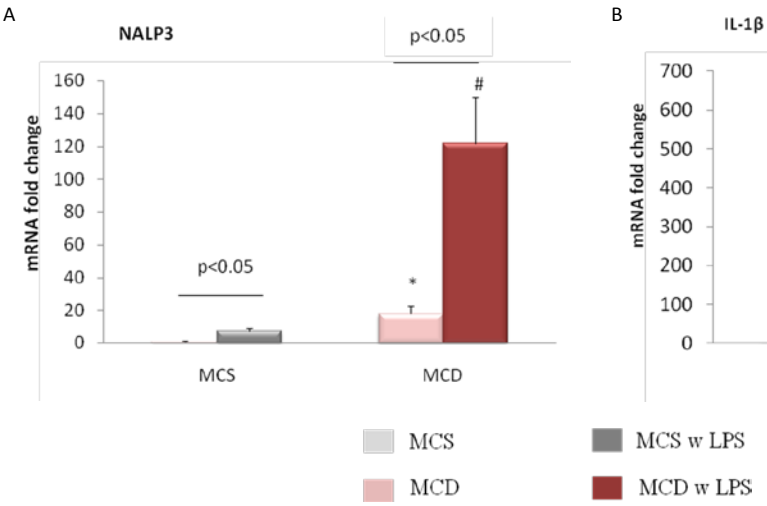

Figure 37. *C57/Bl6 mice were fed with MCD or MCS diet for 5 weeks and injected with LPS ip. for 2 hours. Hepatic NALP3 (A) and IL-1β (B) mRNA expression were analyzed by qPCR, while mature IL-1β protein levels were measured by ELISA (C). N=6 mice/group, (*) indicates p<0.05 vs. MCS at baseline, (#) indicates p<0.05 vs. MCS after LPS stimulation.*

5.2.5 Inflammasome is upregulated in hepatocytes in NASH

The liver is composed of both parenchymal (hepatocytes) and immune cells (macrophages, among others), where hepatocytes represent the majority of the cell populations. Inflammasome expression and activation has been mostly studied in innate immune cells (117); to date the expression and the role of inflammasome in parenchymal liver cells is largely unknown. Here, we sought to evaluate whether inflammasome activation occurs in

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hepatocytes. We found that primary hepatocytes of MCD diet-fed mice showed increased expression of NALP3, ASC, pro-caspase-1, pannexin-1 and pro-IL-1beta mRNA compared to controls *(Figure 38A)*, but not the liver mononuclear cells *(Figure 38B).*

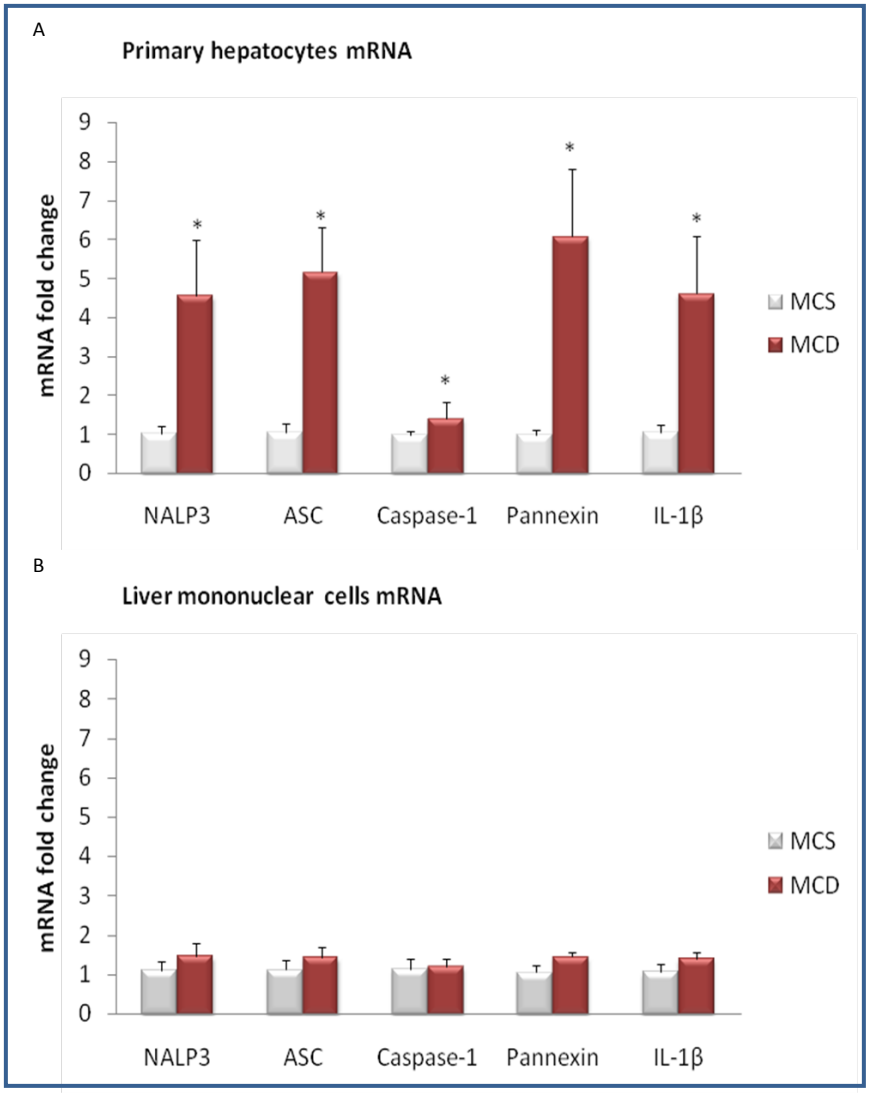

Figure 38. *C57/Bl6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks. Primary hepatocytes and liver mononuclear cells (LMNCs) were isolated as described in the Methods. Hepatocyte (A) and LMNC (B) mRNA of IL-1β and NALP3 inflammasome complex including NALP3, pro-caspase-1, ASC, pannexin-1 was determined.*

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The purity of the primary hepatocyte isolates was confirmed by high expression of albumin and lack of inflammatory cell markers: CD11b (monocytes, macrophages), F4/80 (macrophages), CD11c (dendritic cells), GFAP (stellate cells) *(Figure 39).*

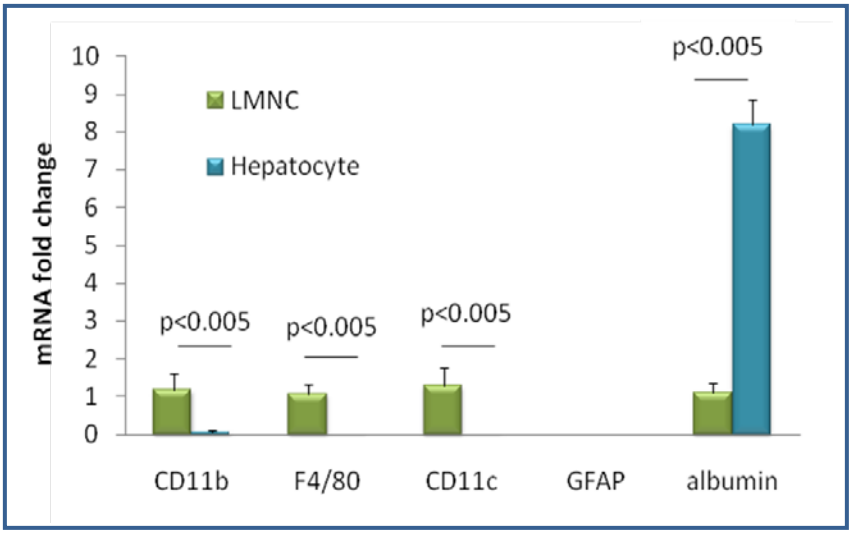

Figure 39. *The purity of the primary hepatocyte isolates was confirmed by measuring mRNA expression of inflammatory cell markers: CD11b (monocytes, macrophages), F4/80 (macrophages), CD11c (dendritic cells), GFAP (stellate cells) and albumin as a hepatocye marker; liver mononuclear cells and total liver were used as controls.*

5.2.6 Fatty acids and LPS induce inflammasome activation in hepatocytes and mononuclear cells

Both circulating fatty acids and gut-derived endotoxins (LPS) contribute to the pathogenesis of NASH (1,56). We found increased serum endotoxin levels in mice with steatohepatitis suggesting that gut-derived LPS, a TLR4 ligand, is present in this model of NASH *(Figure 40)*. We also found that both MCD and HFD diet feeding resulted in significant steatosis indicated by increased hepatic triglyceride levels *(Figure 29, 33).*

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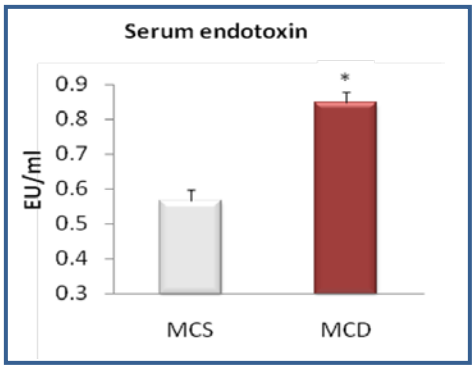

Figure 40. *C57/Bl6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and primary hepatocytes were isolated as described in the Methods. Serum endotoxin is shown as mean±SEM. N=4-6 mice/group.*

Taking into account that fatty livers had elevated expression of inflammasome components *(Figure 27, 31)* and this process occurred in hepatocytes *(Figure 38A)*, next we tested the effects of fatty acids and LPS on inflammasome expression in liver cells. In vitro treatment revealed that palmitic acid, a saturated fatty, acid induced increased expression of NALP3 mRNA in both Hepa 1-6 cells (used as prototypes for hepatocytes) *(Figure 41A)* and in RAW macrophages (used as prototypes for liver macrophages) *(Figure 41B).* In contrast, unsaturated fatty acids, oleic acid *(Figure 41A,B)* or linoleic acid *(Figure 41A,B)* failed to increase the mRNA expression of NALP3 either in Hepa 1-6 cells or RAW macrophages, suggesting that the expression of constitutive inflammasome components is activated exclusively by saturated fatty acids.

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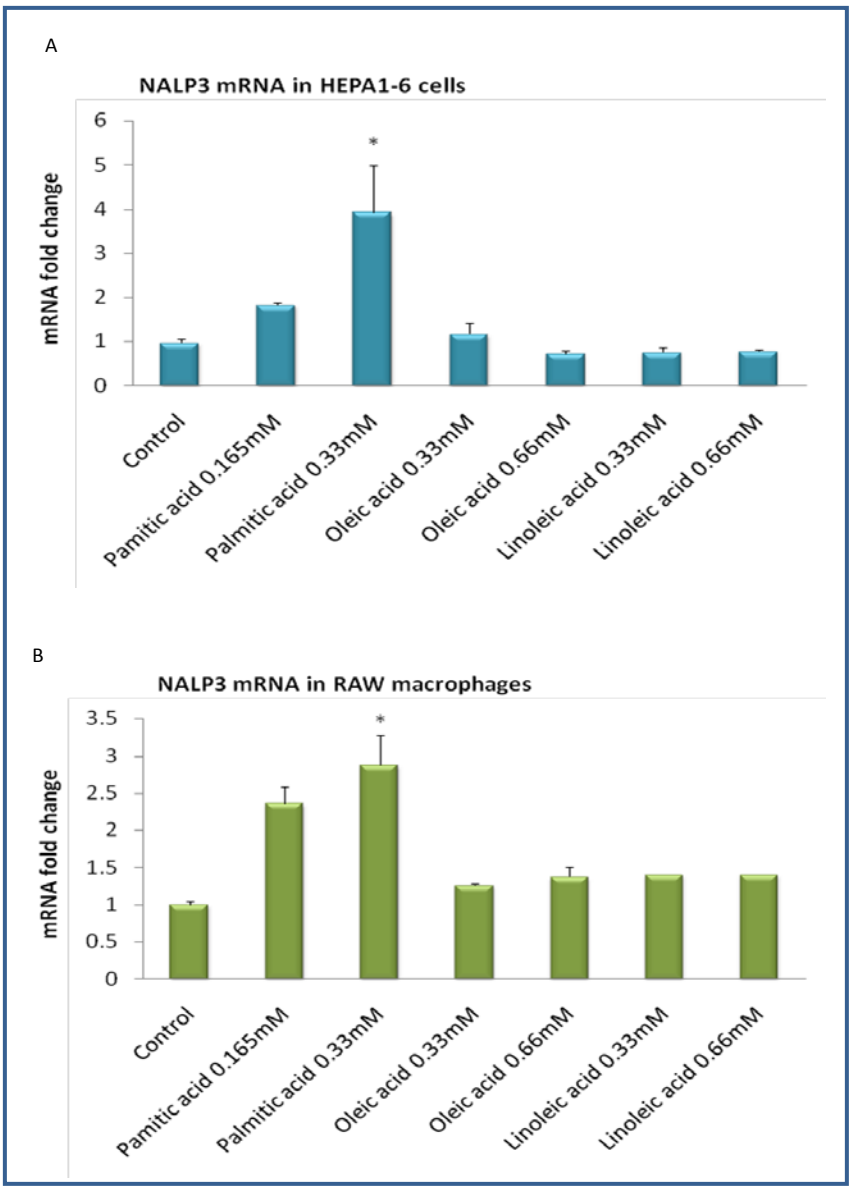

Figure 41. *Hepa 1-6 cells, used as prototypes for hepatocytes (A) and RAW macrophages (B) used as prototypes for macrophages were exposed to satutared fatty acid palmitic acid (0.165mM, 0.33mM), or unsaturated fatty acids, such as oleic acid (0.33mM, 0.66 mM) and linoleic acid (0.33mM, 0.66mM). NALP3 mRNA expression was analyzed by qPCR. (*) indicates $p<0.05$ vs. control.*

Based on the novel observation in the cell line we sought to validate the results in primary hepatocytes. Murine primary hepatocytes were treated with palmitic acid, LPS or their combination (18 hours PA pretreatment followed by LPS). Palmitic acid or LPS alone upregulated NALP3 mRNA expression in hepatocytes ($p<0.01$) *(Figure 42A)* and palmitic

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acid induced moderate increase in IL-1ß protein secretion *(Figure 42B).* Significantly higher levels and earlier IL-1ß production was seen in hepatocytes with palmitic acid pre-treatment followed by LPS stimulation ($p<0.001$) compared to PA or LPS treatment alone suggesting sensitization in hepatocytes *(Figure 42B).*

Together, these results suggested that saturated fatty (palmitic) acid sensitizes the inflammasome to LPS-induced IL-1ß release in hepatocytes.

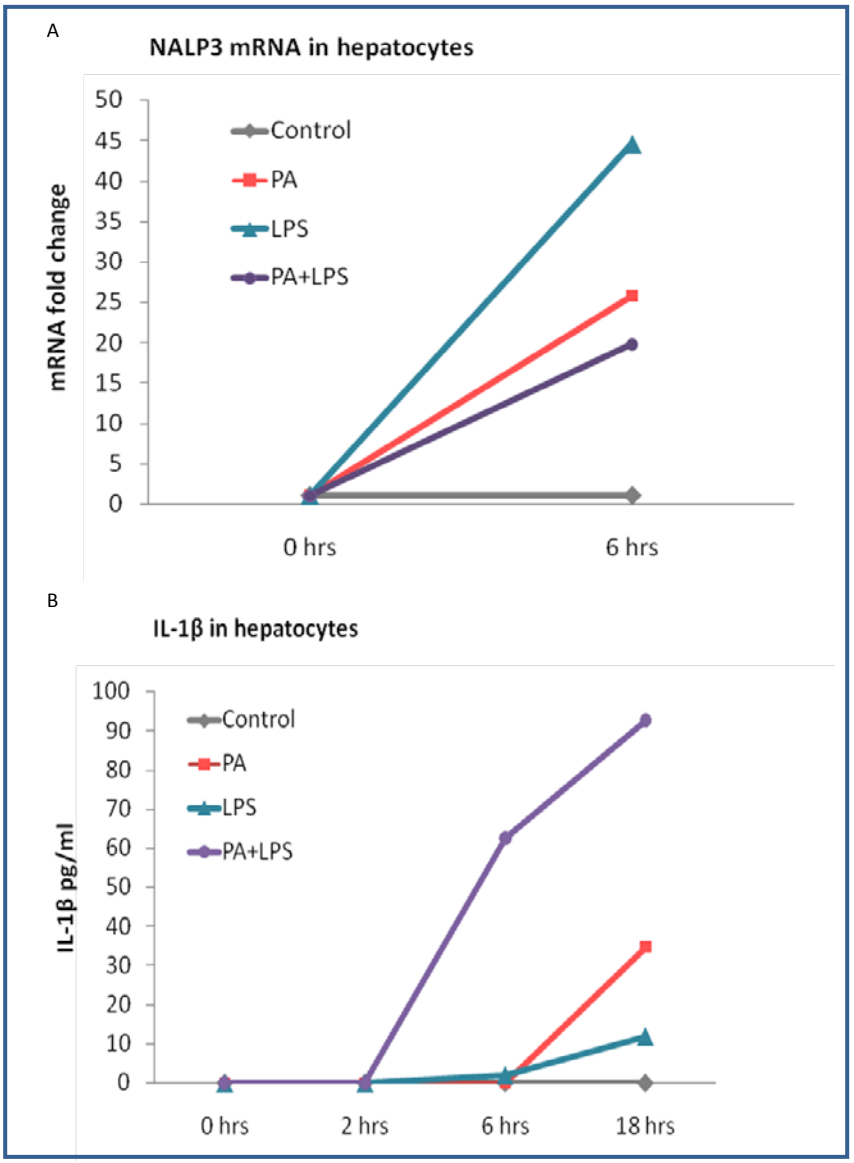

Figure 42. *Isolated hepatocytes from C57/Bl6 mice on normal rodent diet were treated with PA (0.33mM), LPS (1000 ug/ml) or their combination for 6 hours and NALP3 mRNA*

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levels were determined by qPCR (A). IL-1β protein levels in the supernatant of hepatocytes were measured by ELISA following exposure to PA (0.33mM), LPS (1000 ug/ml) or their combination for 2, 6 or 18 hours (B).

5.2.7 IL-1β production in hepatocytes occurs in inflammasome-dependent (caspase-1) and inflammasome-independent (caspase-8-dependent)

To further evaluate the involvement of inflammasome in IL-1ß induction by PA and LPS in hepatocytes, we tested caspase-1 activation that results in cleavage of the 45 kDa procaspase-1 to its enzymatically active form, a heterodimer of p20 and two p10 subunits (117). We found that palmitic acid did not initiate caspase-1 cleavage while pre-treatment with PA followed by LPS stimulation resulted in significant caspase-1 activation in hepatocytes *(Figure 43A).* This pattern of caspase-1 activation mirrored the IL-1ß release after PA pre-treatment and LPS stimulation *(Figure 42B)* suggesting that functional caspase-1 activation in hepatocytes requires signals from both saturated fatty acid and LPS.

The observation that palmitic acid alone induced IL-1ß secretion *(Figure 42B)* without extensive evidence of caspase-1 activation prompted us to evaluate alternative mechanisms for IL-1 cleavage in hepatocytes. While pro-IL-1β cleavage is mostly a result of inflammasome-mediated caspase-1 activation, it can also be cleaved by caspase-8 (193). Indeed, we found that palmitic acid (194,195), but not LPS, resulted in caspase-8 activation and more importantly, caspase-8 activation was not increased by the combination of palmitic acid and LPS. These results suggested that caspase-8 could be involved in the IL-1β cleavage in PA-treated hepatocytes *(Figure 43B).*

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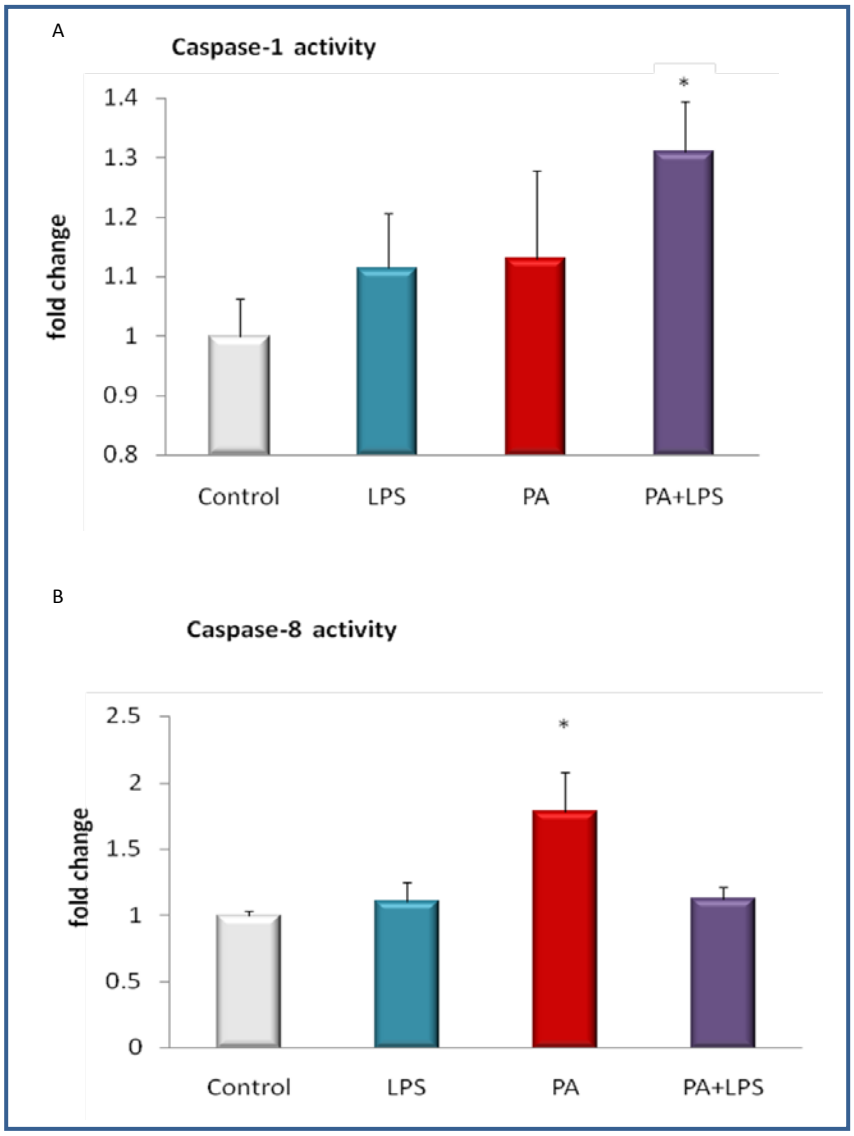

Figure 43. *Isolated hepatocytes from C57/Bl6 mice on normal rodent diet were treated with PA (0.33mM), LPS (1000 ug/ml) or their combination. The activation of the inflammatory caspase-1 and the apoptotic caspase-8 were determined by enzyme activity assay (A: caspase-1 activity, B: caspase-8 activity). (*) indicates p<0.05 vs. control.*

5.2.8 Palmitic acid-treated hepatocytes transmit danger signals and induce inflammasome activation in liver mononuclear cells

In addition to IL-1 cleavage, caspase-8 is also induced in apoptosis (196,197). Our observation of caspase-8 activation by palmitic acid *(Figure 43B)* along with the previous

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reports on induction of apoptosis of hepatocytes by saturated fatty acids (195-197) prompted us to evaluate the mechanistic link between inflammasome activation and cell death in NASH *(Figure 43B, Figure 44).* Increased LDH release in hepatocytes after PA treatment indicated induction of cell death *(Figure 44).*

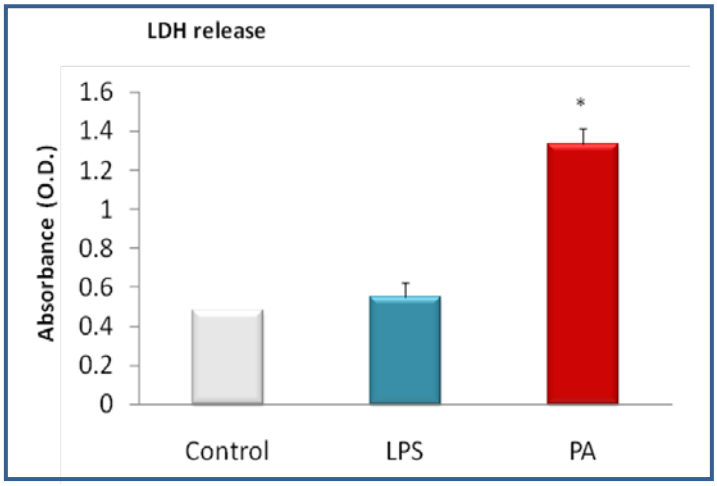

Figure 44. *Isolated hepatocytes from C57/Bl6 mice on normal rodent diet were treated with LPS (1000 ug/ml) or PA (0.33mM) for 18 hours. LDH release as a marker of cell death was determined. (*) indicates $p<0.05$ vs. control.*

We determined that up-regulation of NALP3 and IL-1β mRNA by PA was caspase-dependent because these events were prevented by addition of the pan-caspase inhibitor, ZVAD in hepatocytes *(Figure 45A, 45B).* This observation also suggested that damage-associated molecules generated in apoptotic hepatocytes rather than palmitic acid itself could contribute to inflammasome activation.

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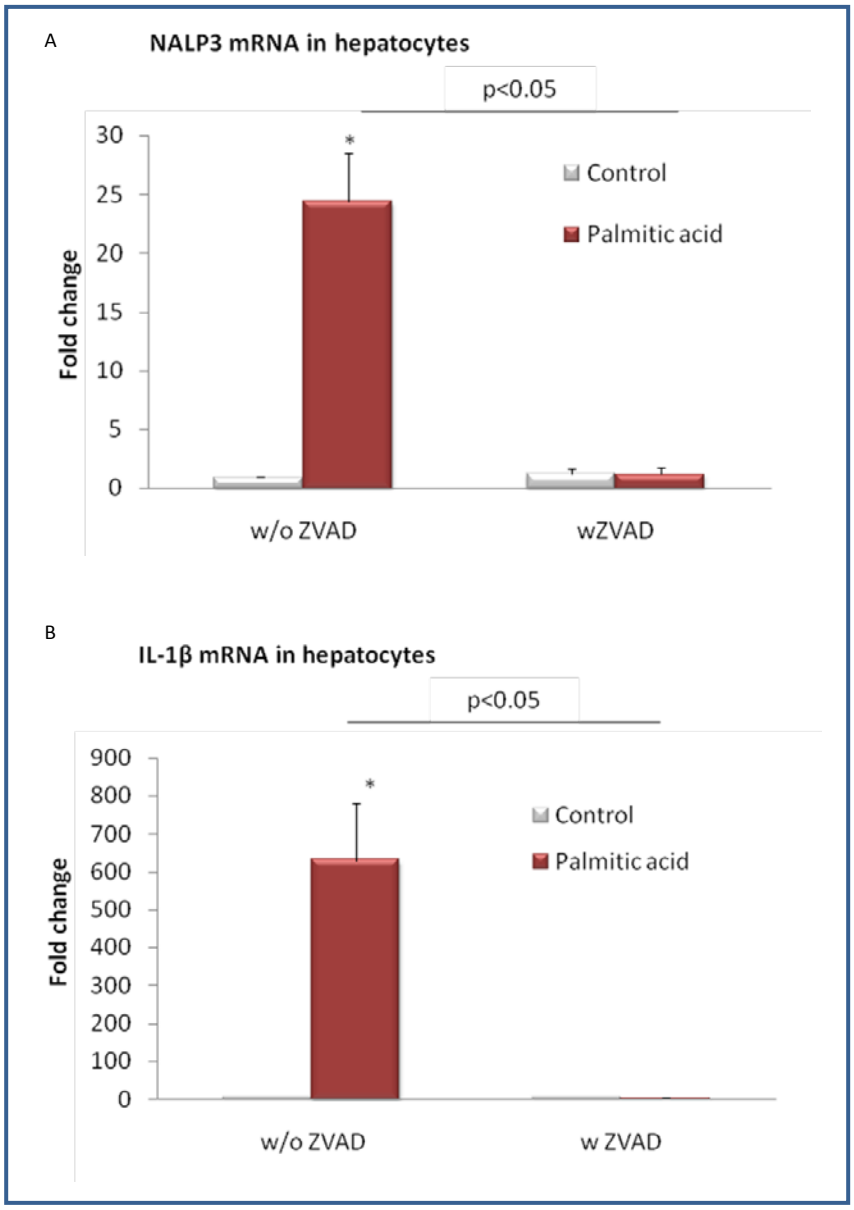

Figure 45. *Isolated hepatocytes from C57/Bl6 mice on normal rodent diet were treated with PA (0.33mM), LPS (1000 ug/ml) in presence or without pancaspase-inhibitor ZVAD (40uM). Hepatocyte mRNA of NALP3 (A) and IL-1β (B) were analyzed by qPCR. (*) indicates p<0.05 vs. control.*

To further evaluate the role of hepatocyte-derived damage-associated molecules in inflammasome activation and a potential cross-talk between hepatocytes and mononuclear cells, we tested whether palmitic acid-treated hepatocytes could induce inflammasome activation in inflammatory cells. Hepatocytes were treated with PA for 6 hours then

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cultured in fresh media without PA. We found that these PA-free supernatants from PA-pretreated hepatocytes induced upregulation of NALP3 *(Figure 46A)* and IL-1β *(Figure 46B)* mRNA in the LMNCs, suggesting that fatty acid-exposed hepatocytes can transfer activation to surrounding immune cells. Transmission of hepatocyte-derived danger signals to MNC was dependent on caspase activation in hepatocytes as suggested by lack of LMNC activation with hepatocyte supernatants when ZVAD was added together with PA to hepatocytes *(Figure 46A,B).*

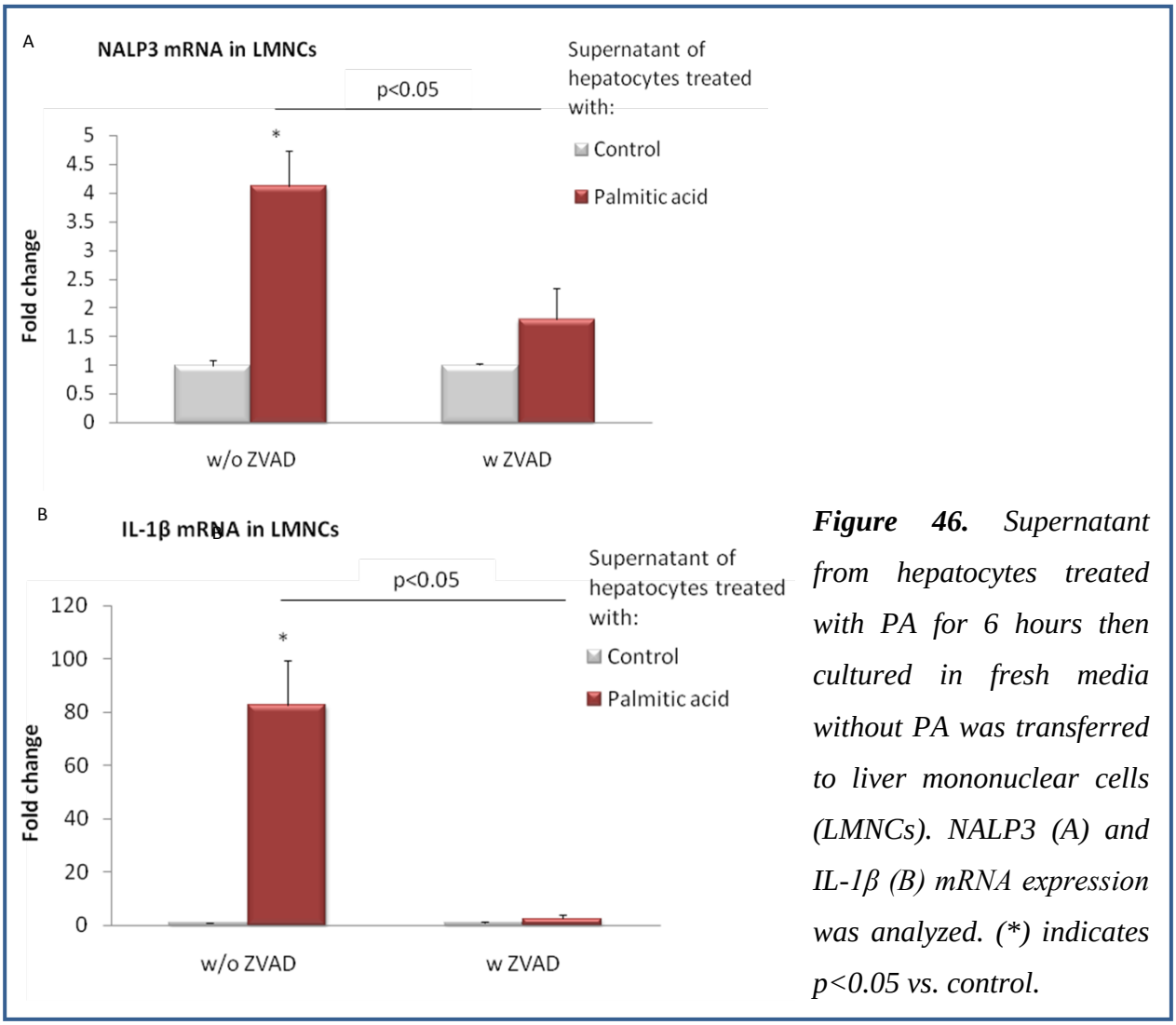

Figure 46. *Supernatant from hepatocytes treated with PA for 6 hours then cultured in fresh media without PA was transferred to liver mononuclear cells (LMNCs). NALP3 (A) and IL-1β (B) mRNA expression was analyzed. (*) indicates p<0.05 vs. control.*

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These results suggested that hepatocytes are the first target of FA and produce inflammasome-mediated danger signals, which in turn activate macrophages in a caspase-dependent manner.

5.2.9 ASC deficiency does not prevent liver injury and fat deposition in the MCD-diet model of NASH

Next step we tested the physiological significance of inflammasome activation in NASH using ASC (apoptosis-associated speck-like protein containing a CARD domain) KO mice. The adaptor molecule ASC is responsible for the formation of inflammasome and bridges the activated NLRs with pro-caspase-1 resulting in caspase-1 activation (117). It has been reported using a cell-free system that ASC is required for caspase-1 activation and IL-1β processing (198). Furthermore, ASC deficiency leads to impaired IL-1β processing in response to LPS in THP-1 cells (199).

In contrast to our primary hypothesis the lack of ASC failed to prevent the development of MCD-diet induced steatohepatitis, as suggested by presence of steatosis on histology *(Figure 47)*, comparable levels of liver triglyceride *(Figure 48)* and serum ALT *(Figure 49)* to the controls.

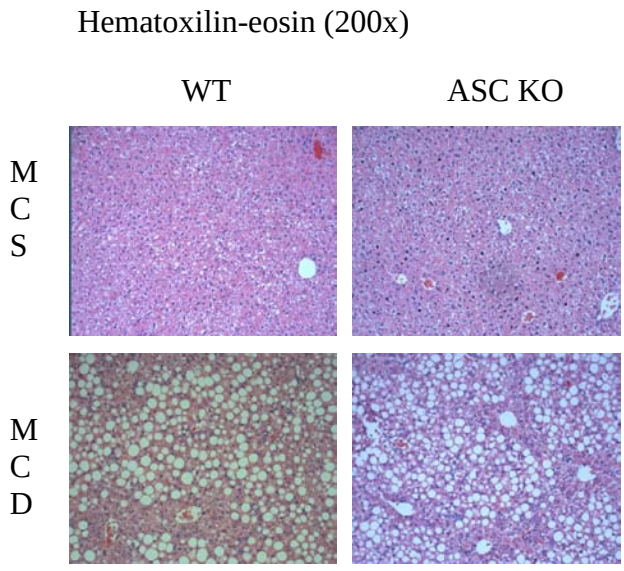

Figure 47. *Wild type and ASC knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Liver tissue was subjected to H&E. One representative slide from n=6/group is shown.*

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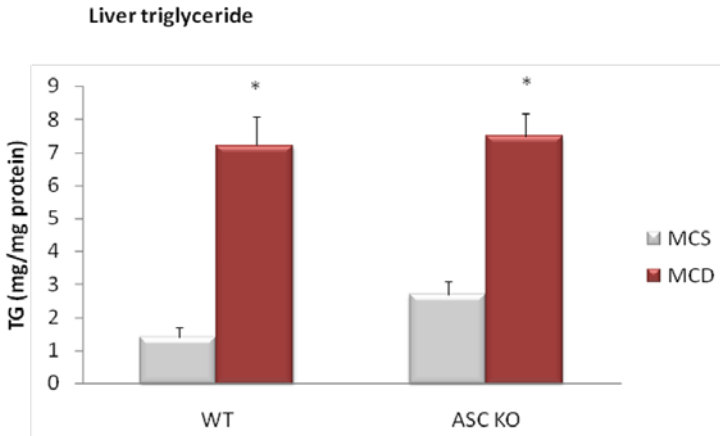

Figure 48. *Wild type and ASC knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Liver triglyceride levels were measured. n=6/group, (*) indicates p<0.05 vs. control*

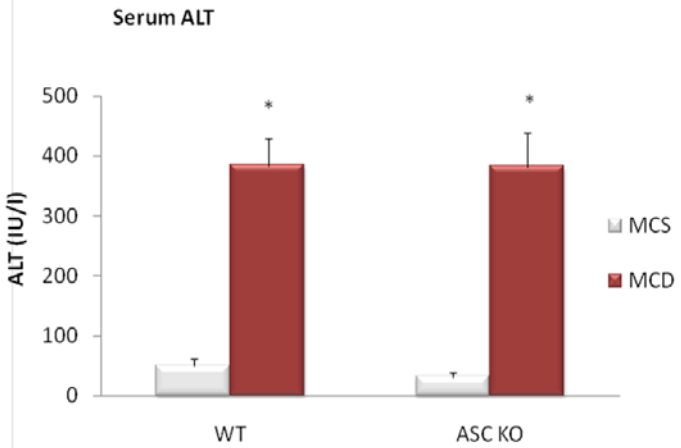

Figure 49. *Wild type and ASC knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Serum ALT levels were measured. n=6/group, (*) indicates p<0.05 vs. control*

In addition, ASC deficiency failed to prevent the production of mature IL-1β *(Figure 50)* in the liver. Moreover, we detected caspase-1 activity *(Figure 51)* in the ASC KO MCD-diet fed mice suggesting that inflammasome activation occurred despite of the lack of the adaptor molecule ASC.

ASC is required for the formation of NALP3 and AIM2 inflammasome, and it is crucial for NLRC4-caused caspase-1 and IL-1β activation (117). The possibility that more than one inflammasome complexes are activated in NASH was raised, and the loss of one inflammasome complex (eg. NALP3) could be compensated by others which do not necessarily require ASC as an adaptor protein (eg. NALP1).

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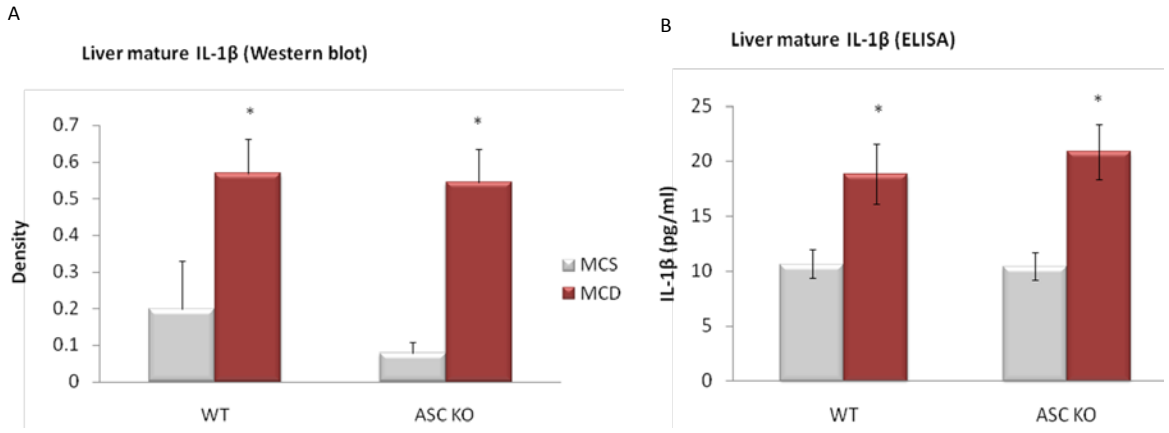


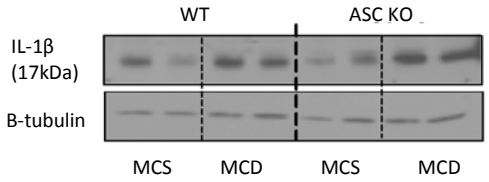
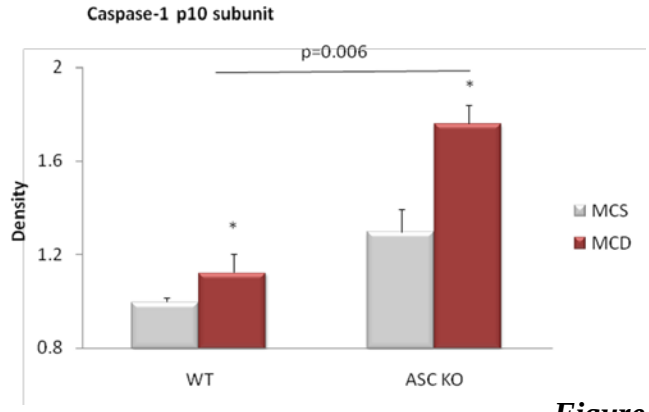


Figure 50. *Wild type and ASC knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Mature (17kDa) IL-1β levels were determined by Western blot (A; top panel: densitometry, bottom panel: a representative Western blot) and by ELISA (B.) n=6/group, (*) indicates p<0.05 vs. control*



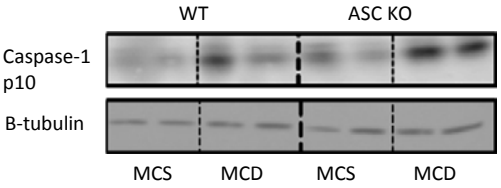


Figure 51. *Wild type and ASC knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. The p10 subunits of the active caspase-1 were detected by Western blot. (*) indicates p<0.05 vs. control*

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5.2.10 Caspase-1 deficiency does not prevent liver injury and fat deposition in the MCD-diet model of NASH

Whether dependent or independent of ASC the formation of the inflammasome complex finally leads to cleavage of pro-caspase-1 to the active enzyme. Therefore, we tested whether the deficiency of caspase-1 attenuates the MCD-diet- induced steatohepatitis.

Surprisingly, the lack of caspase-1 also failed to prevent the MCD-diet-induced steatohepatitis, indicated by the liver histology *(Figure 52)* and the high serum ALT *(Figure 53).* The liver histology showed significant fat accumulation in the caspase-1 KO animals on MCD diet, and the liver triglyceride content was only slightly decreased compared to the WT controls. Furthermore, we found comparable level of hepatic mature IL-1β protein *(Figure 55)* levels in the WT and caspase-1 KO mice. These results were consistent with the data from ASC KO mice *(Figure 47-51)* and suggested that caspases other than caspase-1 may cleave the pro-IL-1β during steatohepatitis.

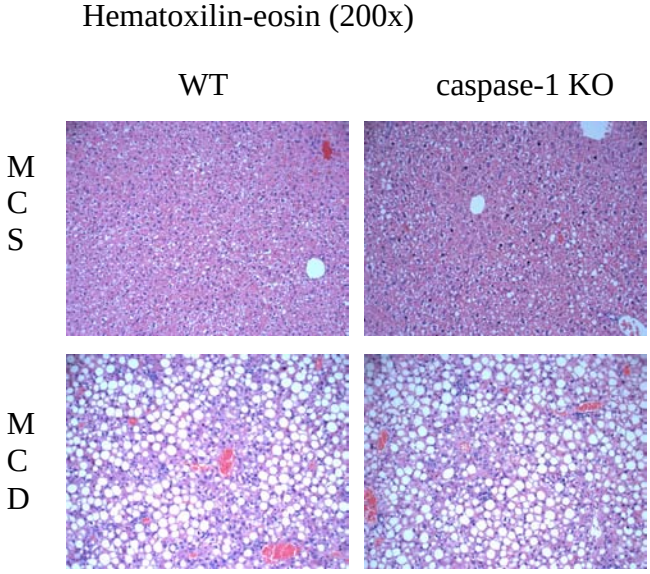

Figure 52. *Wild type and caspase-1 knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Liver tissue was subjected to H&E. One representative slide from n=6/group is shown.*

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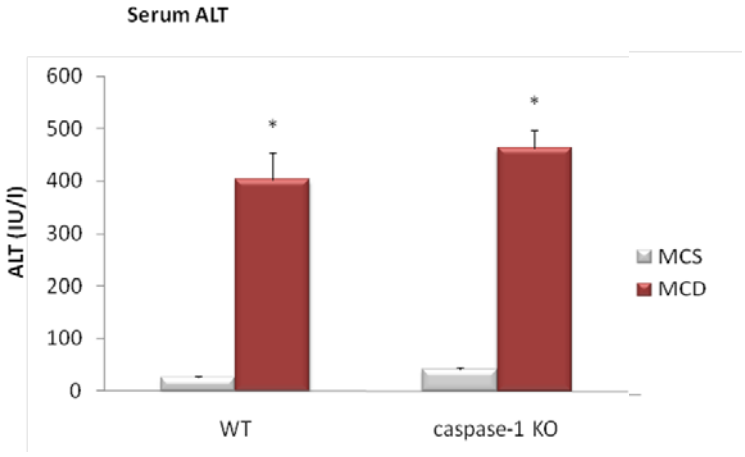

Figure 53. *Wild type and caspase-1 knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Serum ALT levels were measured. n=6/group, (*) indicates p<0.05 vs. control*

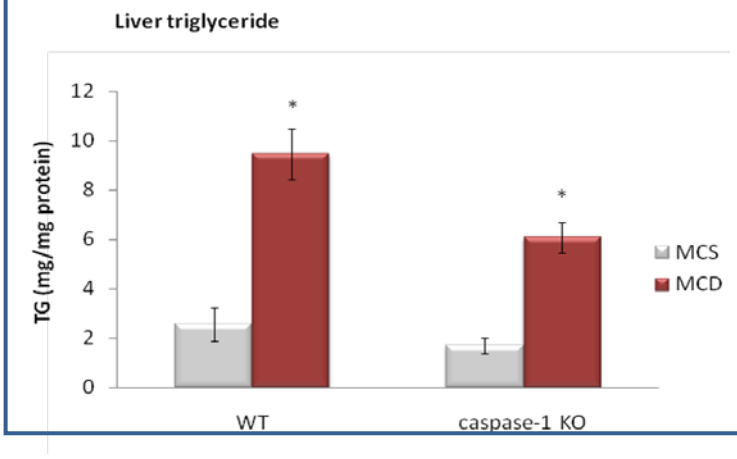

Figure 54. *Wild type and caspase-1 knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Liver triglyceride levels were measured. n=6/group, (*) indicates p<0.05 vs. control*

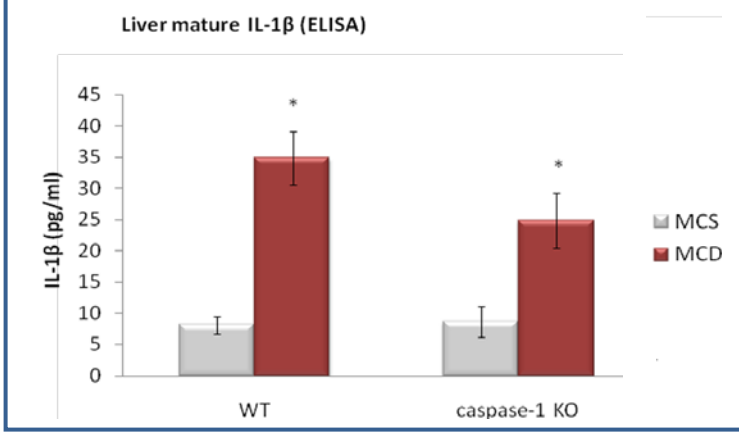

Figure 55. *Wild type and caspase-1 knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Mature (17kDa) IL-1β levels were determined by ELISA. n=6/group, (*) indicates p<0.05 vs. control*

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5.2.11 Caspase-8 as an alternate to cleave IL-1β

Caspase-8 is activated through the extracellular apoptotic pathway, which has been reported crucial during NASH mainly via TRAIL. In addition, caspase-8 is capable to cleave pro-IL-1β in parallel to caspase-1 (193), therefore we tested the levels of caspase-8 activity in our model. Significantly increased caspase-8 activation was found in MCD-diet fed WT mice, which was present at comparable levels both in ASC KO *(Figure 56A,B)* and caspase-1 KO mice *(Figure 57A,B)*. These data suggested that caspase-8 could take over the role of caspase-1, could cleave the pro-IL-1β and thus could explain why ASC or caspase-1 deficiency does not prevent liver injury and steatosis in MCD diet-induced steatohepatitis.

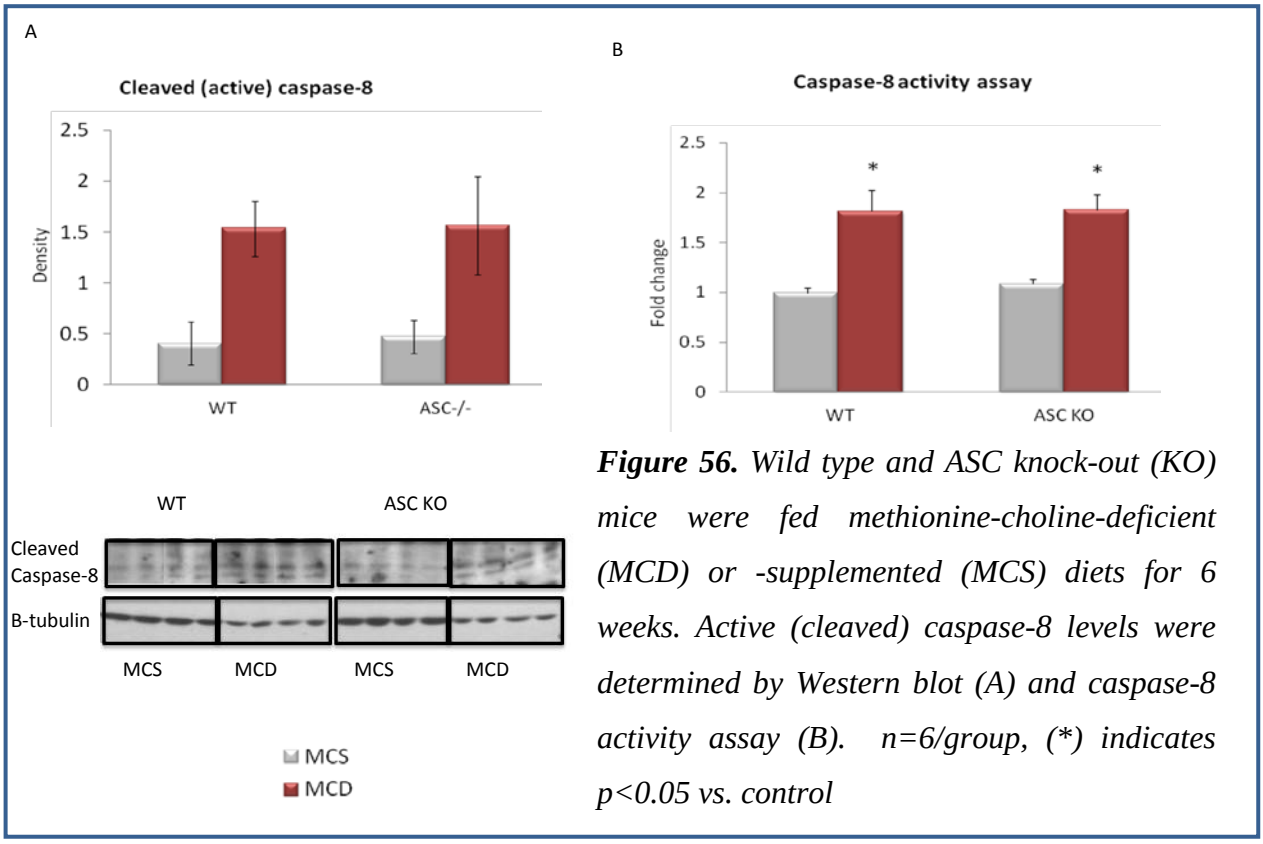

Figure 56. *Wild type and ASC knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Active (cleaved) caspase-8 levels were determined by Western blot (A) and caspase-8 activity assay (B). n=6/group, (*) indicates p<0.05 vs. control*

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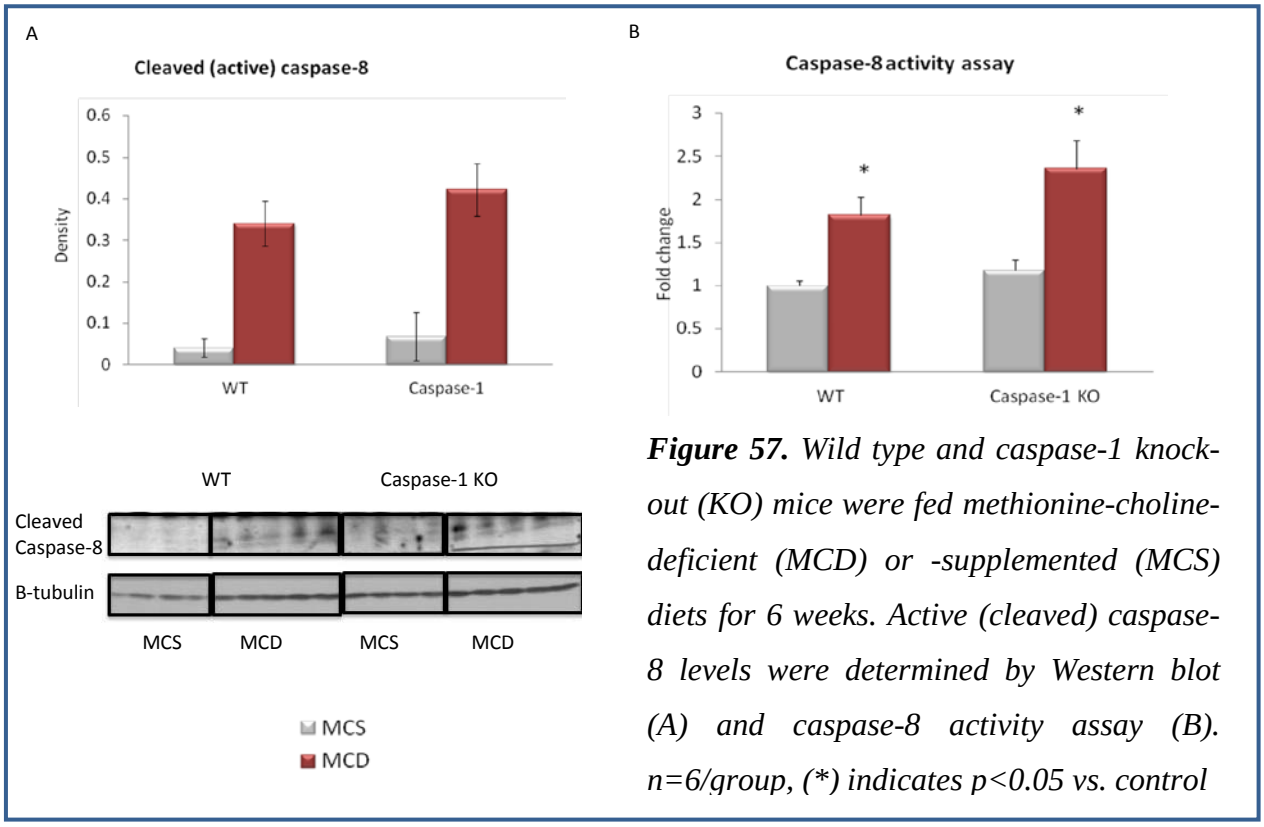

Figure 57. *Wild type and caspase-1 knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 6 weeks. Active (cleaved) caspase-8 levels were determined by Western blot (A) and caspase-8 activity assay (B). n=6/group, (*) indicates p<0.05 vs. control*

5.2.12 Interleukin-1 receptor deficiency attenuates hepatic steatosis, but does not prevent MCD-diet-induced liver injury or fibrosis

Interleukin-1β is sensed by the interleukin-1 receptor (IL-1R), therefore to examine the role of IL-1β in MCD-diet induced steatohepatitis, we fed WT and IL-1R KO mice with MCD or MCS diet for 8 weeks. Recently, *Miura et al.* reported that IL-1R deficiency reduced liver injury, steatosis and fibrosis in another, choline-deficient (CD) model of steatohepatitis. Consistently we found attenuated steatosis in MCD-diet fed IL-1R KO mice compared to WT controls, indicated by the liver histology *(Figure 58)* and liver triglycerides *(Figure 59).* However, in contrast to the previous findings in CD-diet induced

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steatohepatitis, IL-1R deficiency failed to prevent liver injury in MCD-diet-fed mice. The serum ALT levels *(Figure 60)* were even slightly higher in the IL-1R KO mice compared to WT controls on MCD-diet. In addition, IL-1R deficiency also failed to prevent the development of liver fibrosis induced by MCD-diet (Sirius Red staining: *Figure 61*, Alpha-smooth muscle actin /αSMA/ immunohistochemistry: *Figure 62*, collagen mRNA: *Figure 63*)

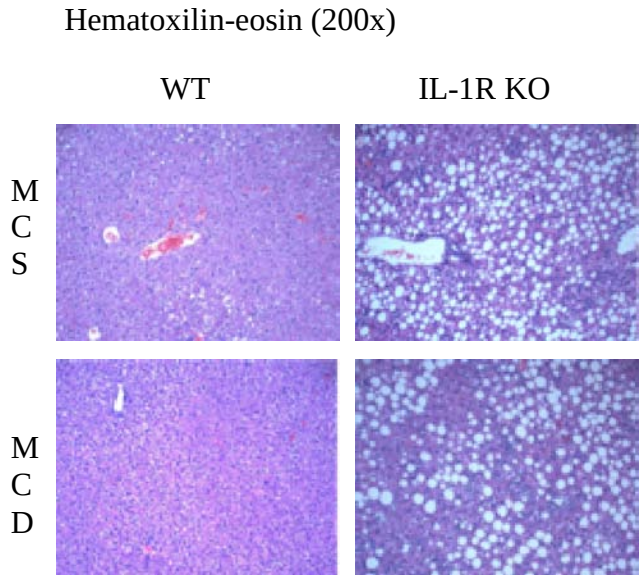

Figure 58. *Wild type and IL-1R knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 8 weeks. Liver tissue was subjected to H&E. One representative slide from n=6/group is shown.*

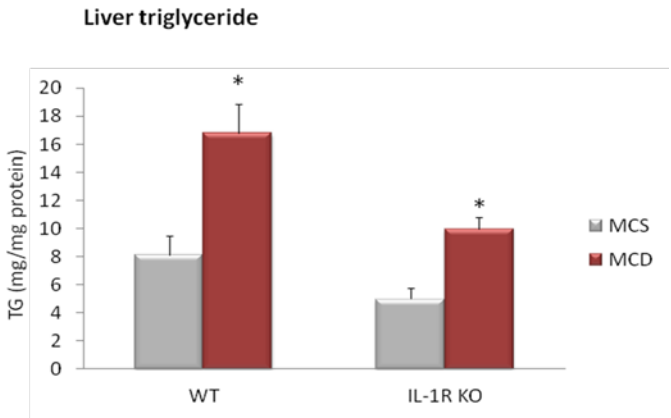

Figure 59. *Wild type and IL-1R knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 8 weeks. Liver triglyceride levels were measured. n=6/group, (*) indicates p<0.05 vs. control*

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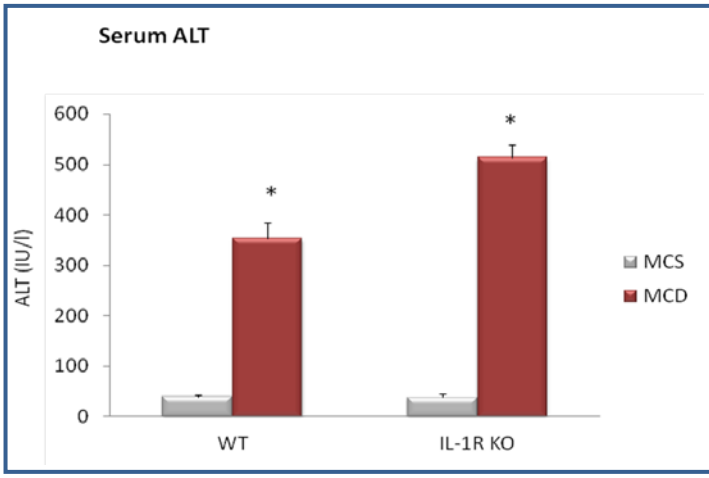

Figure 60. *Wild type and IL-1R knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 8 weeks. Serum ALT levels were measured. n=6/group, (*) indicates p<0.05 vs. control*

Sirius Red staining

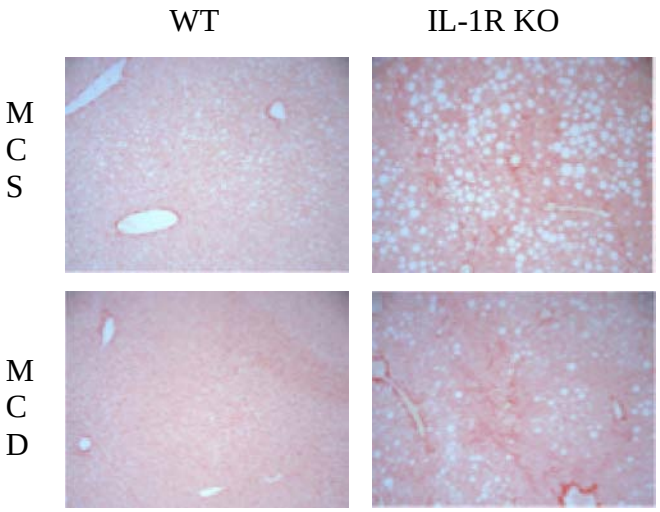

Figure 61. *Wild type and IL-1R knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 8 weeks. Liver tissue was subjected to Sirius Red staining. One representative slide from n=6/group is shown.*

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Alpha smooth muscle actin (αSMA)immunohistochemistry

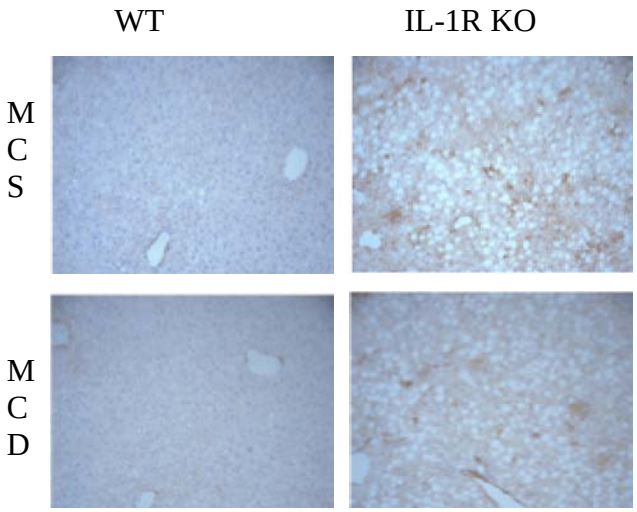

Figure 62. *Wild type and IL-1R knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 8 weeks. Liver tissue was subjected to αSMA immunohistochemistry. One representative slide from n=6/group is shown.*

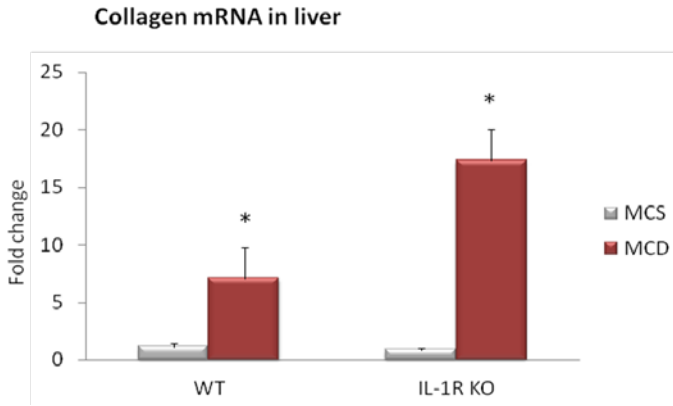

Figure 63. *Wild type and IL-1R knock-out (KO) mice were fed methionine-choline-deficient (MCD) or -supplemented (MCS) diets for 8 weeks. Collagen mRNA levels were measured by PCR in the liver. n=6/group, (*) indicates p<0.05 vs. control*

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5.3 Mitochondrial antiviral signaling protein defect links impaired antiviral response and liver injury in steatohepatitis in mice

5.3.1 Type-I IFN induction is decreased in steatohepatitis in response to poly I:C stimulation

Polyinosinic-polycytidylic acid (poly I:C), a synthetic double-stranded RNA (dsRNA), is a surrogate for viral infection (200). Double stranded RNA is recognized by TLR3 and helicase receptors and induces robust Type-I IFN response leading to anti-viral immunity (102). Antiviral responses to RNA are important in HCV and HIV infection (51,201). Here we show for the first time that poly I:C-induced Type-I interferon production is significantly decreased in mice with steatohepatitis *(Figure 64)*. We found decreased serum protein *(Figure 64A)* and liver mRNA levels *(Figure 64B)* of IFNβ and IFNα4 *(Figure 64C)* in MCD compared to MCS diet-fed control mice. Consistent with impaired Type-I IFN production after poly I:C stimulation, induction of interferon-inducible genes ISG56 *(Figure 65A)* and ISG15 *(Figure 65B)*, was also significantly decreased in MCD diet-induced steatohepatitis. These results suggested that steatohepatitis results in impaired Type-I IFN response to dsRNA viral challenge.

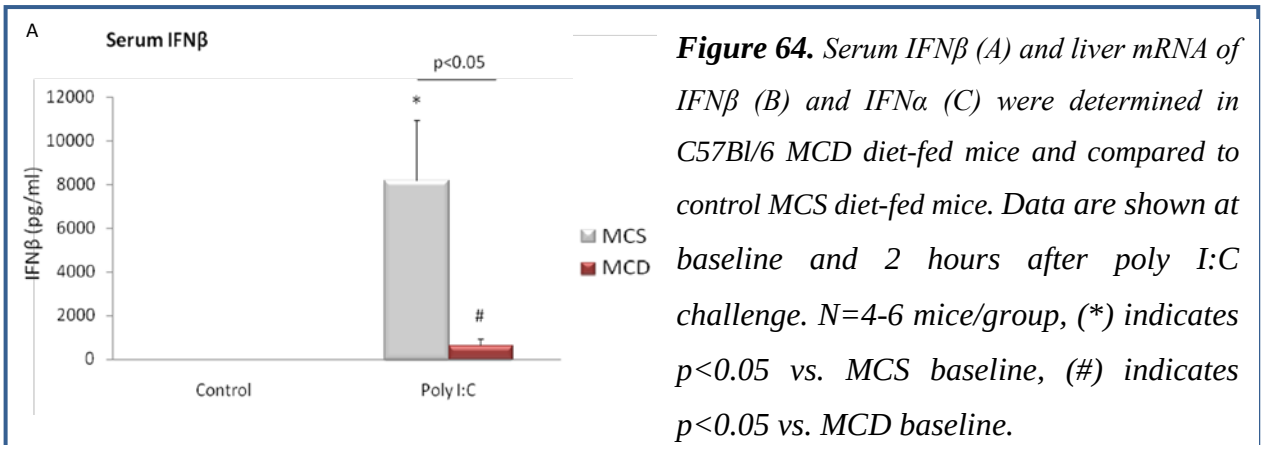

Figure 64. *Serum IFNβ (A) and liver mRNA of IFNβ (B) and IFNα (C) were determined in C57Bl/6 MCD diet-fed mice and compared to control MCS diet-fed mice. Data are shown at baseline and 2 hours after poly I:C challenge. N=4-6 mice/group, (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

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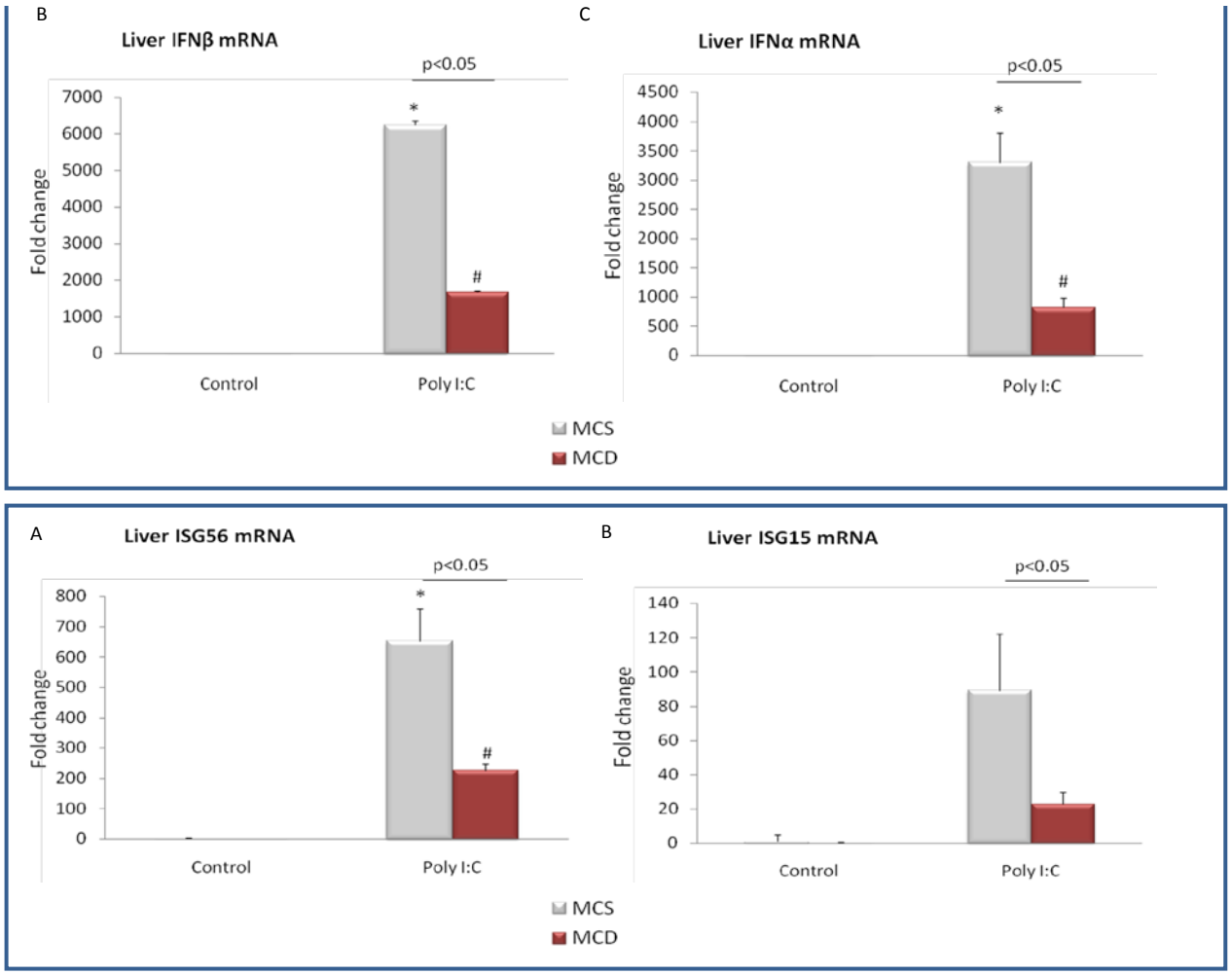

Figure 65. *Liver mRNA of IFN-inducible genes, ISG56 (A) and ISG15 (B), were determined in C57Bl/6 MCD diet-fed mice and compared to control MCS diet-fed mice. Data are shown at baseline and 2 hours after poly I:C challenge. N=4-6 mice/group, (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

5.3.2 Impaired Type-I IFN induction in steatohepatitis is restricted to the RIG-I/Mda5 pathway

To further evaluate the significance of impaired Type-I IFN induction in steatohepatitis, we employed stimulations that induce Type-I IFNs via receptor pathways

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different from dsRNA recognition by TLR3 and its adapter, TRIF, or RIG-I/Mda5 and their adapter MAVS, respectively (102). LPS is recognized by TLR4 and uses the adapters TRIF and MyD88, while CpG DNA, a ligand for TLR9 solely utilizes the MyD88 adapter in Type-I IFN induction (102).

We found increased TLR3, Mda5, RIG-I, as well as their corresponding adapters, TRIF and MAVS at the mRNA levels in fatty livers compared to controls *(Figure 66)*. In contrast to polyI:C, challenge with a TLR4-ligand (LPS), which uses TRIF, or a TLR9-ligand (CpG DNA), which uses MyD88, resulted in increased Type-I IFN induction in MCD compared to MCS diet-fed mice *(Figure 67A,B,C).* TRIF serves as sole adapter for poly I:C-engaged TLR3 and it also mediates TLR4/LPS-induced Type-I IFN production (102). TRIF deficient mice were shown to be defective in both TLR3 and TLR4 mediated IRF3 activation (202). These data suggested a selective impairment of Type-I IFN induction upon dsRNA viral (poly I:C) challenge in a TLR3/TRIF-independent manner; we thus focused on dissecting the role of the helicase RNA-sensing pathways in steatohepatitis.

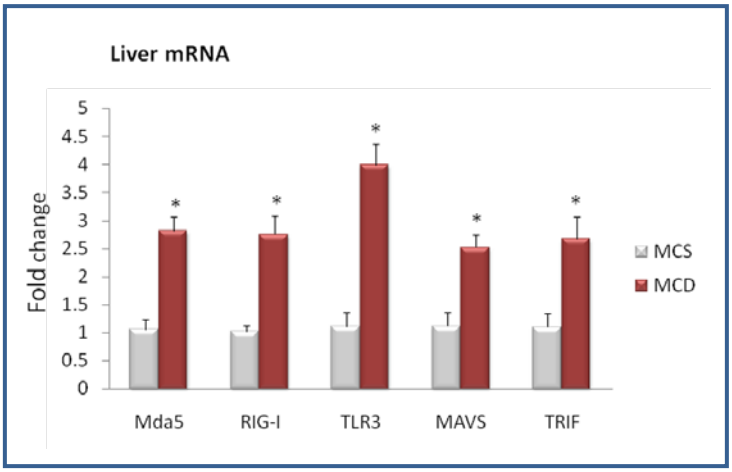

Figure 66. *The mRNA expression of poly I:C (dsRNA) sensing receptors, namely Mda5, RIG-I, TLR3 and their adaptor molecules, MAVS and TRIF, respectively, were measured by qPCR in the liver of MCD and MCS diet-fed mice. N=4-6 mice/group, (*) indicates p<0.05 vs. MCS.*

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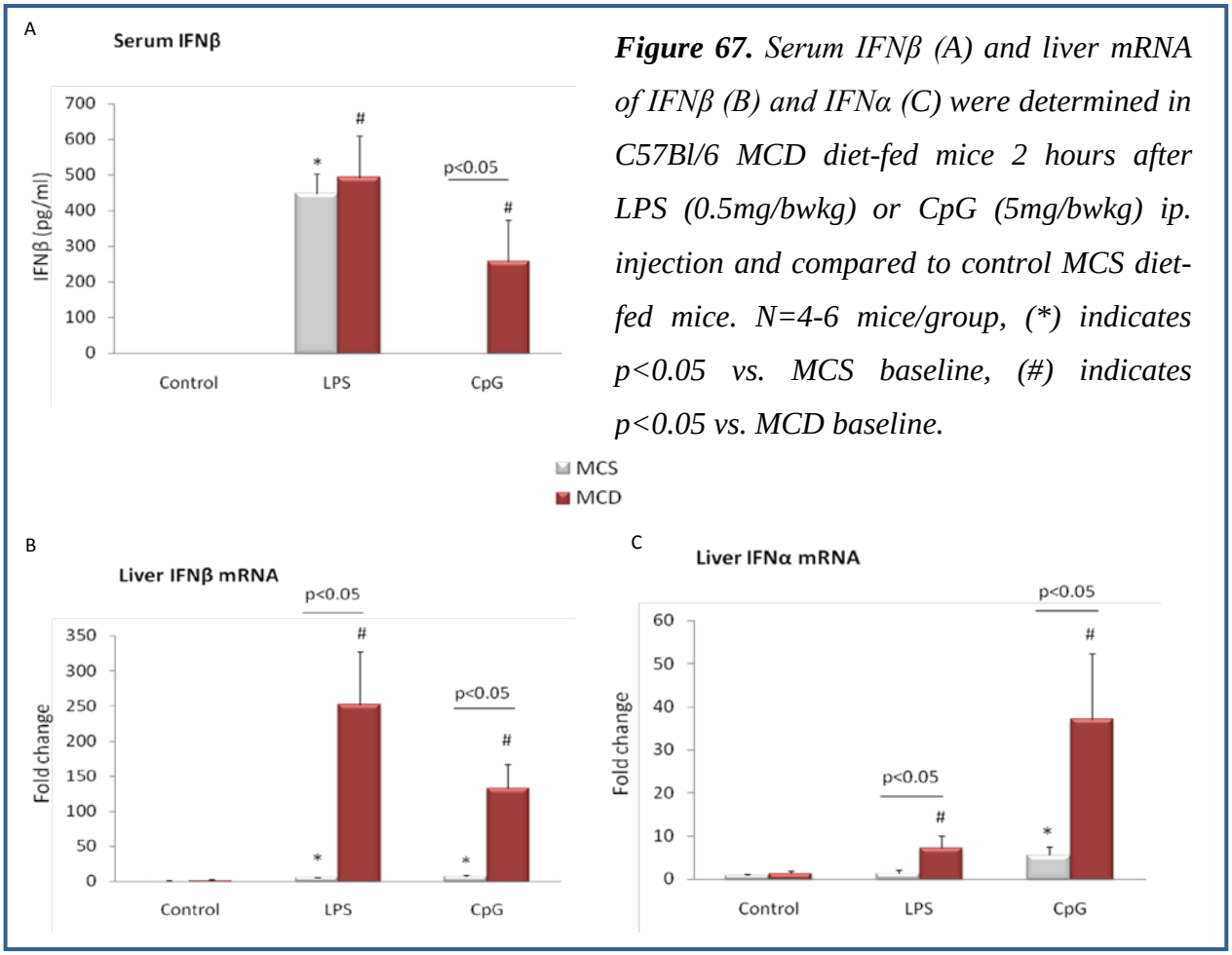

Figure 67. *Serum IFNβ (A) and liver mRNA of IFNβ (B) and IFNα (C) were determined in C57Bl/6 MCD diet-fed mice 2 hours after LPS (0.5mg/bwkg) or CpG (5mg/bwkg) ip. injection and compared to control MCS diet-fed mice. N=4-6 mice/group, (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

5.3.3 Abnormal MAVS function in NASH involves decreased protein levels, dissociation from the mitochondria and impaired oligomerization

The adapter molecule MAVS is critical for the downstream signaling of helicase receptors and its dysfunction impairs proinflammatory cytokine and interferon induction via the NFκB and IRF3 signaling pathways, respectively (105). Consistent with decreased induction of Type-I IFN, we found decreased levels of MAVS protein in whole liver lysates of MCD-diet fed mice compared to controls *(Figure 68).* In search of possible mechanisms for decreased MAVS protein levels, we found higher mRNA expression of

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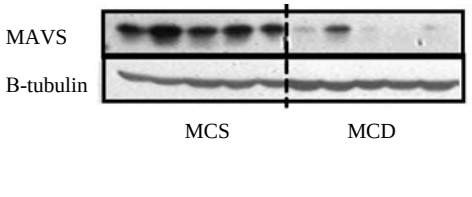
the PSMA7 subunit of proteasome in MCD steatohepatitis *(Figure 69A).* PSMA7 can negatively regulate MAVS-mediated immune responses and promotes proteosomal degradation (203). Immunoprecipitation experiments revealed increased association between MAVS and PSMA7 in fatty livers compared to controls *(Figure 69B).*



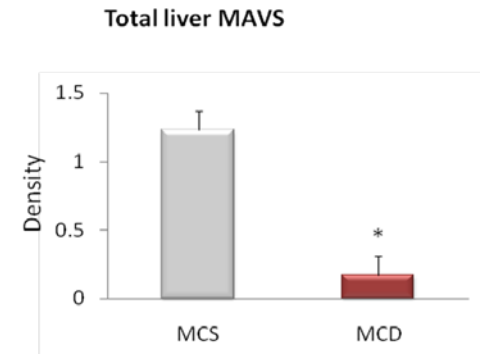


Figure 68. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks. MAVS protein expression was analyzed by Western blot in liver whole cell lysates (A); β-tubulin was used as loading control (A). (*) indicates p<0.05 vs. MCS.*

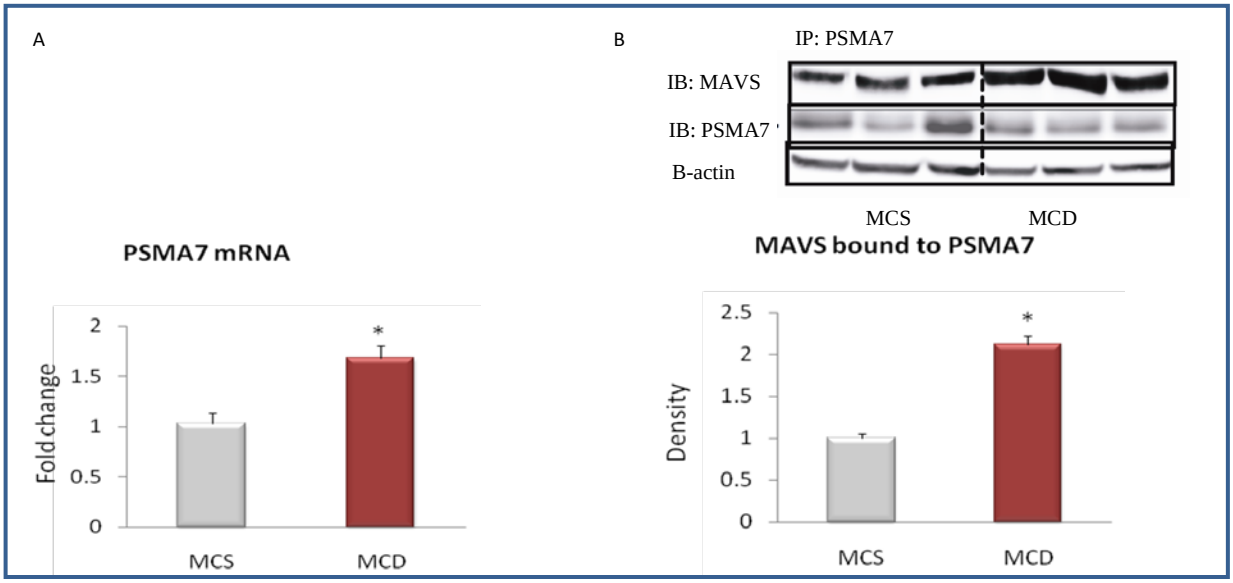


Figure 69. PSMA7 mRNA expression was measured in the liver of MCD or MCS diet-fed mice (A). PSMA7 associated MAVS expression was evaluated by immunoprecipitation in whole liver lysates using PSMA7 antibody for immunoprecipitating and MAVS antibody for immunoblotting (B). N=4-6 mice/group, (*) indicates p<0.05 vs. MCS.

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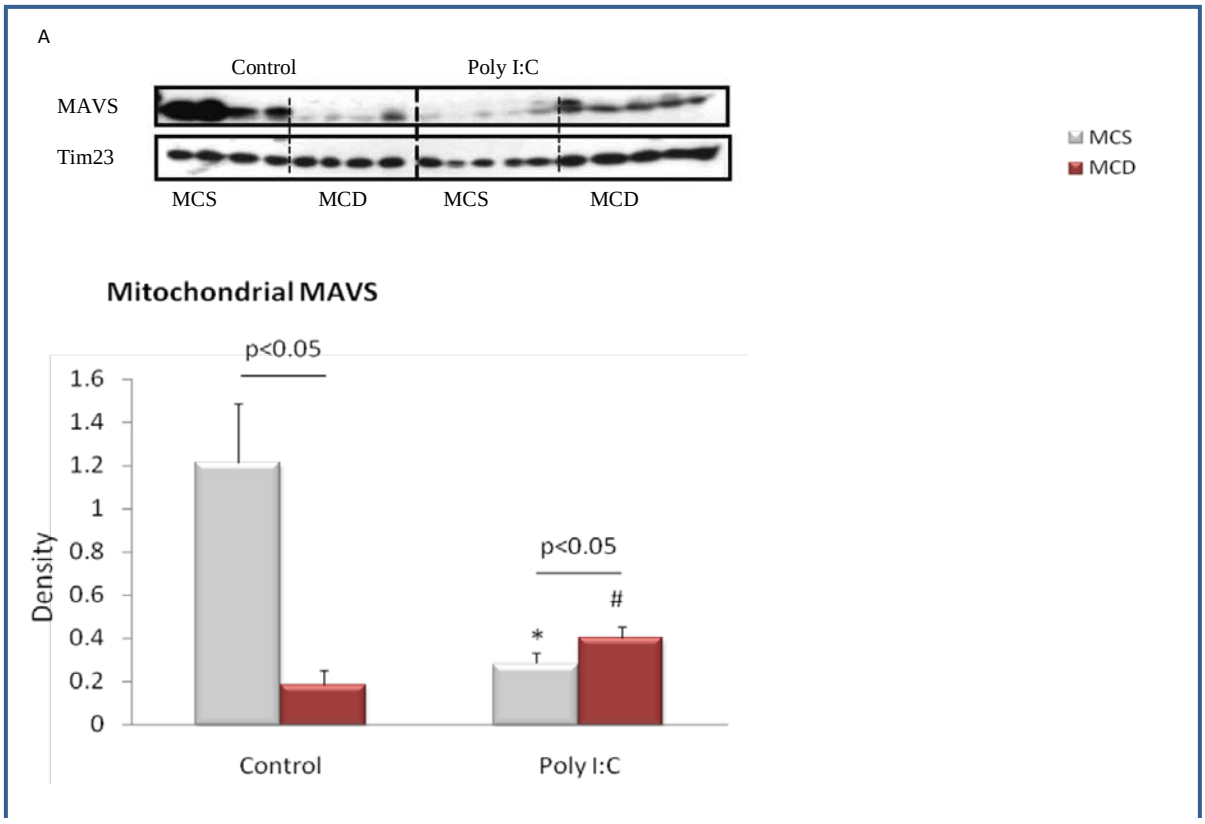
The localization of MAVS to the outer mitochondrial membrane is crucial for Mda5/RIG-I activation (106). However, we found that steatohepatitis resulted in decreased mitochondria-associated MAVS protein levels compared to controls *(Figure 70A).* We also observed a corresponding increase in cytosolic MAVS protein levels in MCD compared to the MCS-diet fed livers *(Figure 70B).* The purity of the mitochondrial and cytosolic preparations was confirmed by the expression of mitochondrial marker Tim23 *(Figure 70A)* and cytosolic β-tubulin *(Figure 70B),* respectively. The ratio of the cytoplasmic/mitochondrial MAVS was significantly higher in MCD-steatohepatitis *(Figure 70C).* These results indicated that displacement of MAVS protein from the mitochondria to the cytosol is likely related to mitochondrial damage in steatohepatitis. The transmembrane domain (TM) of MAVS is crucial for mitochondrial localization and also for dimerization of MAVS that is required for downstream signaling (105,107).



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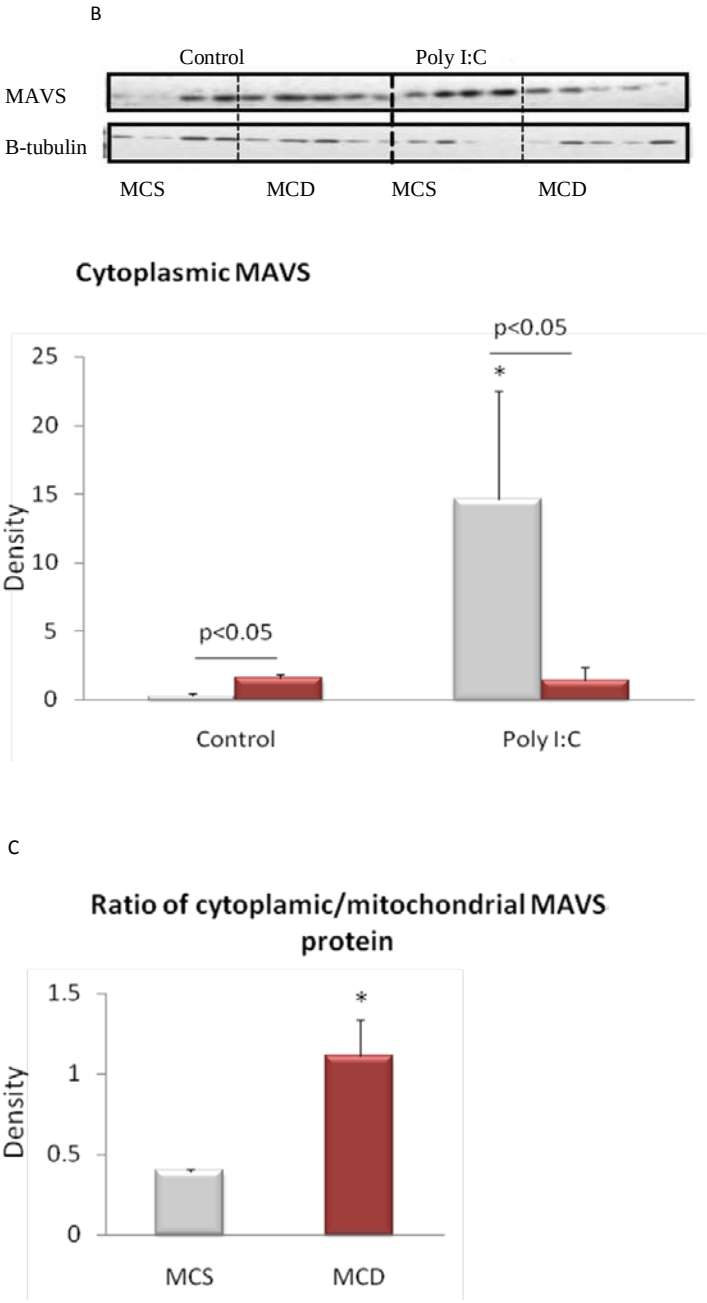

Figure 70. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and injected with poly I:C (5mg/bwkg) intraperitoneally for 2 hours. MAVS protein expression was analysed by Western blot in liver mitochondrial (A) and cytoplasmic extract (B);Tim23 was used as loading control in mitochondrial extract, while β-tubulin was used as loading control in the cytoplasmic extract (A, B, respectively). The ratio of cytoplasmic/mitochondrial MAVS protein levels is shown at panel C. (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

We found that in addition to impaired mitochondrial localization, there was decreased oligomerization of MAVS in steatohepatitis compared to controls *(Figure 71).*

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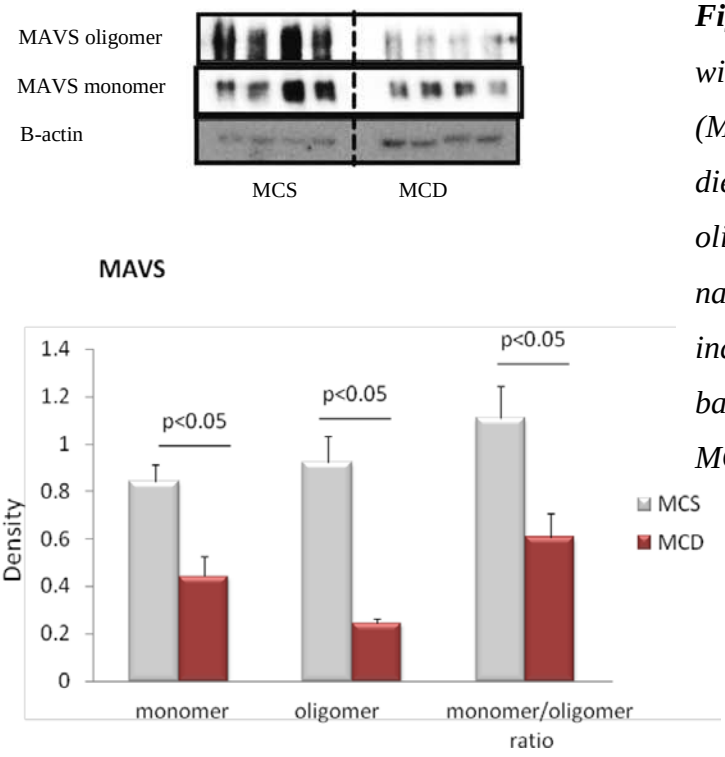

Figure 71. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks. MAVS oligomerization was analyzed on native gel electrophoresis. (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

Given the defects in poly I:C-triggered interferon induction in steatohepatitis *(Figure 64)*, we next explored the function of the MAVS adapter protein. In control mice, poly I:C administration resulted in displacement of MAVS from the mitochondria to the cytosol *(Figure 70).* In contrast, there was no increase in cytoplasmic MAVS translocation after poly I:C stimulation in livers of MCD diet-fed mice *(Figure 70)*. PolyI:C-induced engagement of helicases and signaling through MAVS results in downstream activation and phosphorylation of IRF3 (102). In livers of MCD diet-fed mice, impaired MAVS function and decreased mitochondrial association was associated with significantly reduced IRF3 phosphorylation after poly I:C stimulation *(Figure 72)*. These data suggested that decreased association of MAVS with mitochondria at baseline may impair downstream signaling in stetohepatitis.

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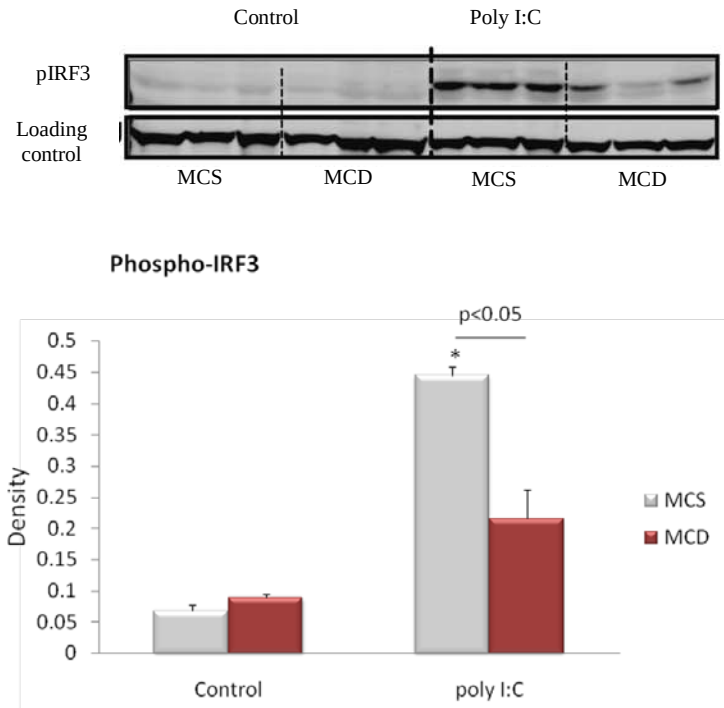

Figure 72. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and injected with poly I:C (5mg/bwkg) intraperitoneally for 2 hours. IRF3 activation was evaluated by detection of phosphoIRF3 by Western blot in liver whole cell lysates. (*) indicates p<0.05 vs. MCS baseline.*

5.3.4 Mitochondrial damage occurs in the fatty liver

Mitochondrial dysfunction plays a role in the pathogenesis of NASH (204) and upon mitochondrial damage, its content leaks into the cytosol triggering diverse signaling pathways, including apoptosis (205). Thus, we hypothesized that decreased association of MAVS with mitochondria may be linked to mitochondrial damage in NASH. Indeed, mitochondrial damage was indicated by relocation of cytochrome C from the mitochondria to the cytoplasm *(Figure 73A),* and by enrichment of the mitochondria with ß-actin *(Figure 73B)* in livers of MCD compared to MCS diet-fed mice. We further identified evidence for increased cellular damage pathways in steatohepatitis as indicated by caspase 8 *(Figure 74A)* and caspase 1 *(Figure 74B)* activation. Relevant to our observation of decreased MAVS in steatohepatitis, both caspase 8 and caspase 1 were shown to cleave MAVS from the mirochondria (206,207,208).

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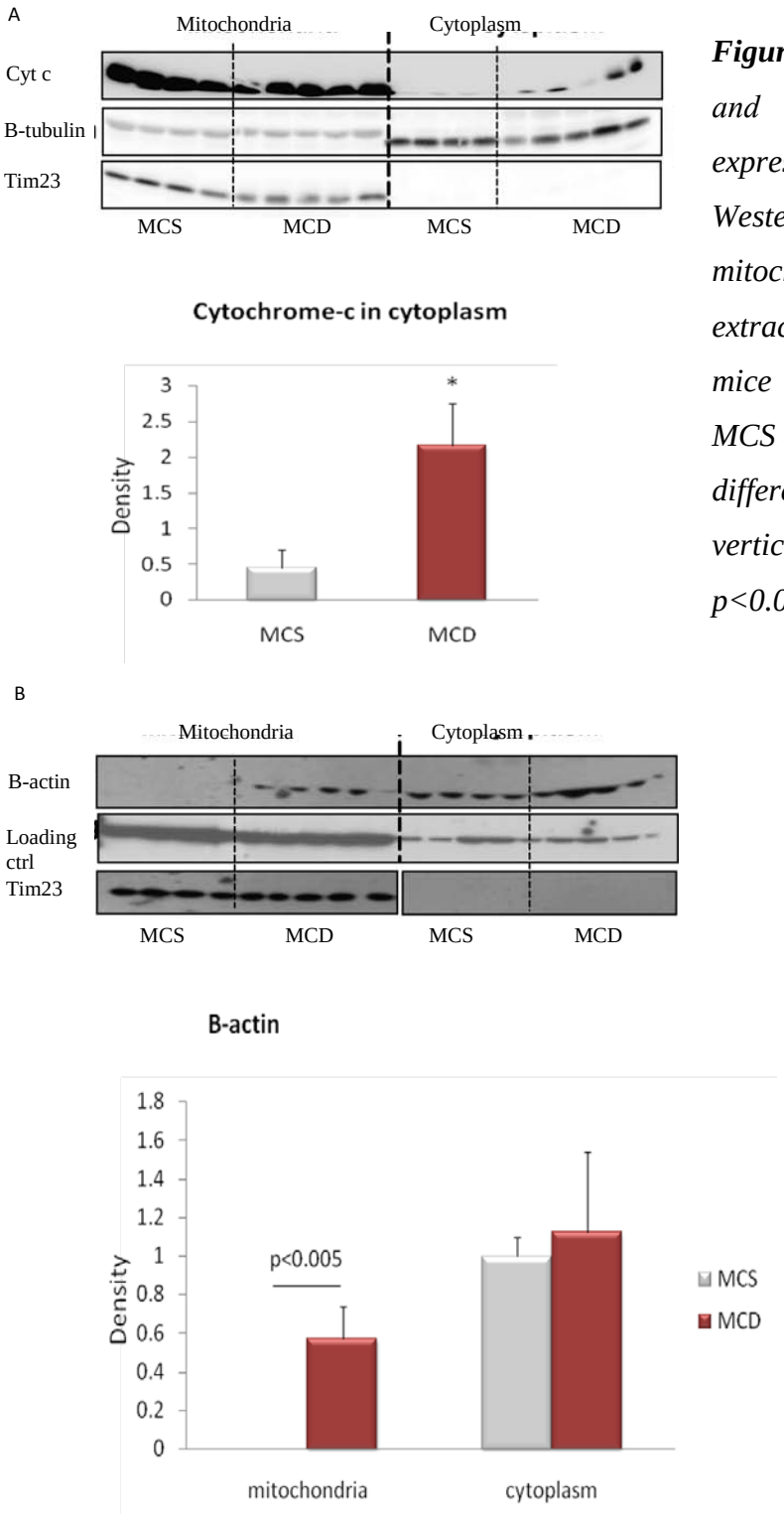

Figure 73. *Cytochrome c (A) and β-actin (B) protein expressions were analyzed by Western blot in liver mitochondrial and cytoplasmic extract of C57Bl/6 MCD diet-fed mice and compared to control MCS diet-fed mice. Lanes run on different gels are separated by vertical white line. (*) indicates $p<0.05$ vs. MCS baseline.*

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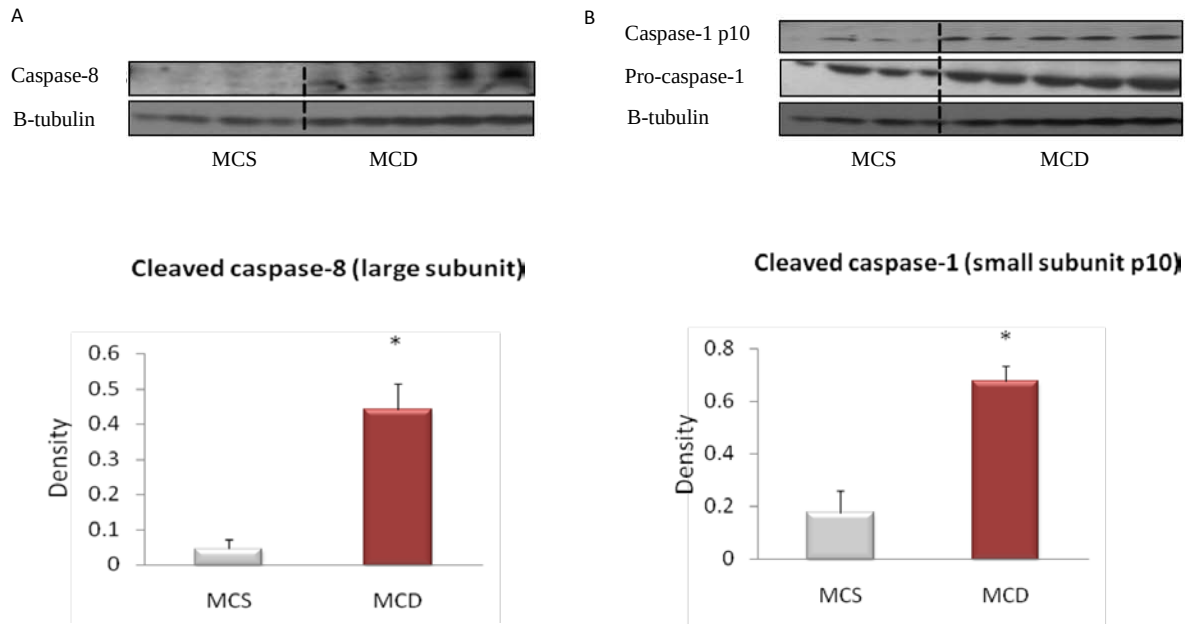

Figure 74. *The activation of apoptotic caspase-8 (A) and inflammatory caspase-1 (pro-caspase and p10 subunit) (B) were determined by Western blot analysis in liver whole cell lysates of C57Bl/6 MCD diet-fed mice and compared to control MCS diet-fed mice using specific antibodies. β-tubulin was used as loading control. (*) indicates p<0.05 vs. MCS baseline.*

Mitochondrial damage in NASH has been linked to excessive levels of reactive oxygen species (ROS) (204). Indeed, we detected significantly increased liver TBARs levels indicating ROS-induced lipid peroxidation at baseline and after poly I:C stimulation in steatohepatitis *(Figure 75)*. These results indicated that ROS and lipid peroxidation occur in NASH, and their production is exacerbated in response to dsRNA stimulation.

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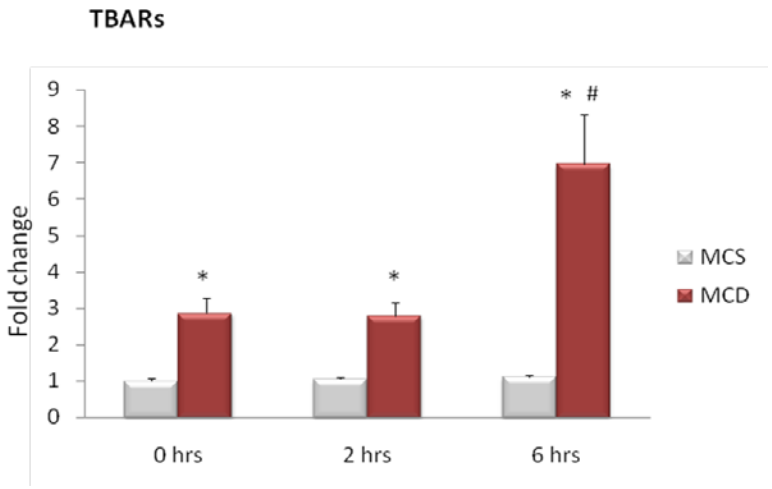

Figure 75. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and injected with poly I:C (5mg/bwkg) intraperitoneally for 2 and 6 hours. Liver thiobarbituric acid (TBARs) levels were analyzed as indirect indicators of ROS production, n=5-6/group (E). (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

5.3.5 Increased poly I:C-induced liver damage occurs without excessive pro-inflammatory cytokine induction in steatohepatitis

Liver damage, indicated by steatosis and elevated ALT, is a hallmark of steatohepatitis. Here we found that a poly I:C challenge significantly increased liver injury in MCD diet-fed mice indicated by tissue hemorrhage, hepatocyte degeneration *(Figure 76A)*, and significantly increased serum ALT levels compared to MCS controls mice *(Figure 76B)*. Because dsRNA-induced activation of RIG-I and Mda5 leads to Type-I IFN induction as well as activation of NFκB and production of pro-inflammatory cytokines (102), we sought to evaluate whether the increased liver damage was the consequence of enhanced pro-inflammatory cytokine production in steatohepatitis. At baseline, MCD diet-fed mice showed increased serum *(Figure 77A)* and liver mRNA levels *(Figure 77B)* of TNFα, IL-6 and IL-1β compared to MCS controls. While poly I:C challenge increased TNFα, IL-6 and IL-1β production both in controls and MCD-diet fed groups *(Figure 77A,B)*, the extent of pro-inflammatory cytokine protein *(Figure 77A)* and mRNA *(Figure*

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77B) induction was significantly lower in MCD compared to MCS diet-fed mice. These data demonstrated that pro-inflammatory cytokine induction was impaired in response to a dsRNA challenge, and thus, it is less likely to account for the increased liver damage in NASH.

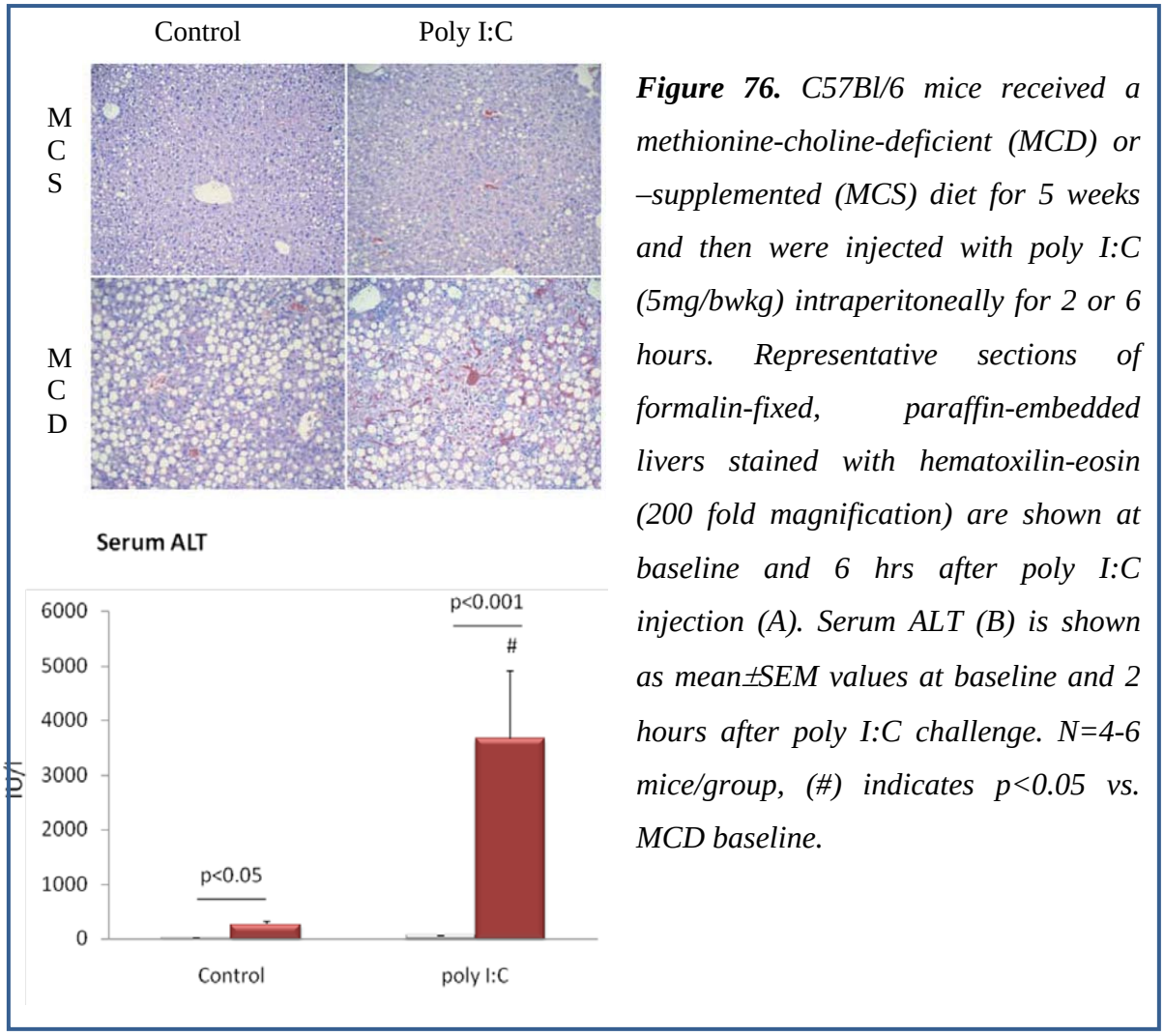

Figure 76. *C57Bl/6 mice received a methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and then were injected with poly I:C (5mg/bwkg) intraperitoneally for 2 or 6 hours. Representative sections of formalin-fixed, paraffin-embedded livers stained with hematoxilin-eosin (200 fold magnification) are shown at baseline and 6 hrs after poly I:C injection (A). Serum ALT (B) is shown as mean±SEM values at baseline and 2 hours after poly I:C challenge. N=4-6 mice/group, (#) indicates p<0.05 vs. MCD baseline.*

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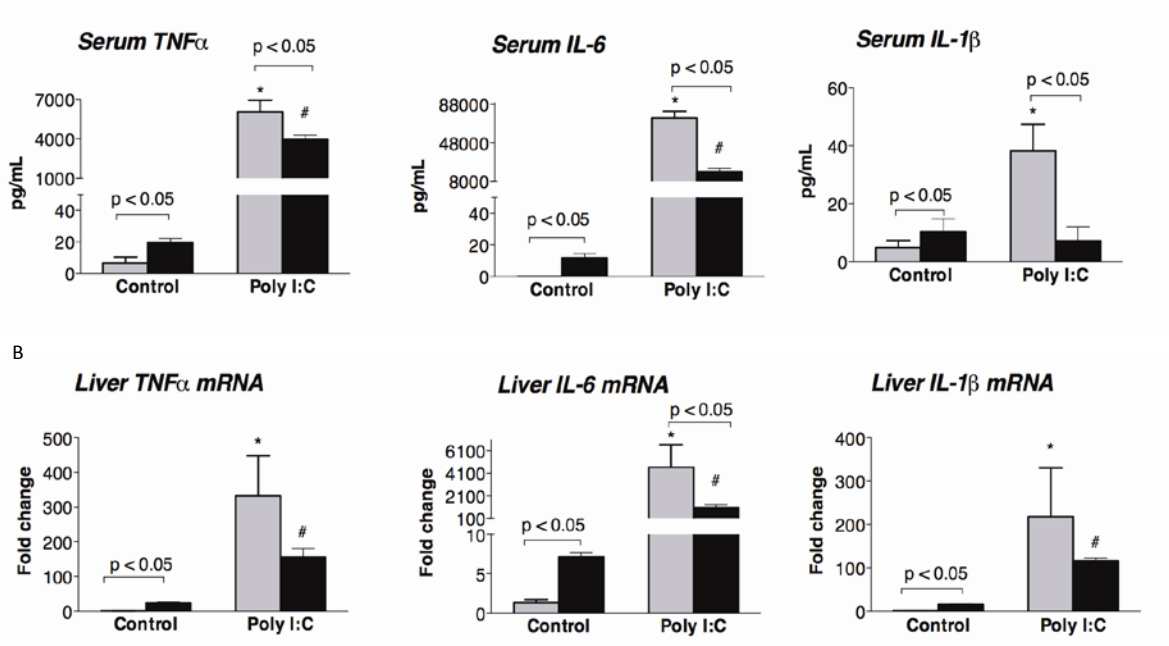

Figure 77. *C57Bl/6 mice received a methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and then were injected with poly I:C (5mg/bwkg) intraperitoneally for 2 or 6 hours. Serum protein (A) and liver mRNA (B) of cytokines (TNFα, IL-6, IL-1β) are shown as mean±SEM values at baseline and 2 hours after poly I:C challengeN=4-6 mice/group, (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

Since, previous studies showed a crucial role for NK cells in poly I:C induced liver injury (209), and higher NK cell activating ligand expression has been reported in livers of NASH patients (210), we next investigated the possible role of NK cells. We found increased mRNA expression of the NK-activating ligands, Pan-Rae, Rae-1α and Mult-1 in MCD-steatohepatitis *(Figure 78)*, but poly I:C did not induce a further increase in the expression of these ligands *(Figure 78).*

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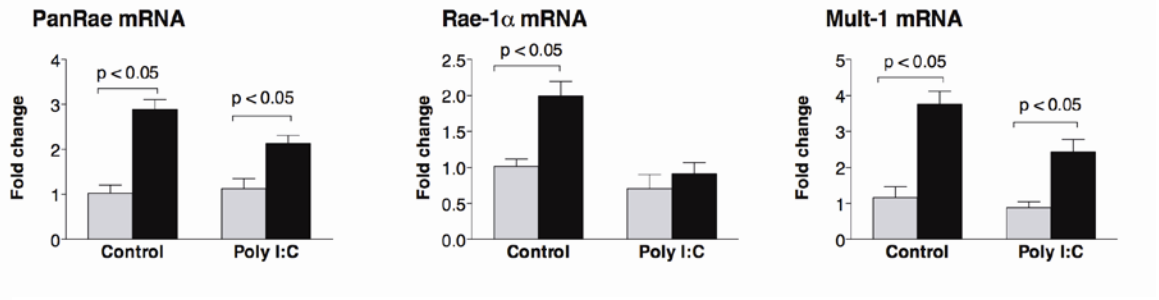

Figure 78. *C57Bl/6 mice received a methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and then were injected with poly I:C (5mg/bwkg) intraperitoneally for 2 or 6 hours. Liver mRNA expression of NK cell activating ligands was analysed by qPCR (A). N=4-6 mice/group, (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

5.3.6 Poly I:C promotes a switch from apoptosis to necrosis and increases RIP3 expression in steatohepatitis

Hepatocyte apoptosis in NASH has been linked to increased susceptibility of the fatty liver to LPS challenge while hepatocyte necrosis is associated with progressive liver damage (44). There is recent evidence that the mitochondria-associated MAVS can regulate apoptosis in viral infection (211). Apoptosis is triggered via intrinsic (involving pro-apoptotic protein Bim, mitochondria, cytochrome c, and caspase 9), or via extrinsic (involving death receptors including TRAIL) pathways that connect at the level of caspase 3 to culminate in cell death. We found increased expression of TRAIL (extrinsic apoptosis) *(Figure 79A)* and Bim (intrinsic apoptosis) *(Figure 79B)* in livers of MCD diet-fed mice. Expression of caspase-3 was also induced in MCD vs. MCS diet-fed mice *(Figure 79C)*. Here we found that caspase-3 activity was significantly increased by poly I:C in normal (MCS) livers *(Figure 79C)*, but not in steatohepatitis (MCD) *(Figure 79C)*. There were no differences in the extent of poly I:C-induced upregulation of TRAIL and Bim mRNA expression *(Figure 79A,B)*, between MCD and MCS livers indicating that steatotic livers, while exhibit higher apoptosis at baseline, fail to progress to tissue death by apoptosis upon a viral challenge. Notably, the tissue damage was higher in poly I:C-challenged steatotic

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livers compared to controls *(Figure 76A)*. Thus, we hypothesized that the increased poly I:C-induced liver damage in MCD diet-fed mice was due to necrosis rather than apoptosis. Indeed, we identified increased levels of serum HMGB1 *(Figure 80)*, a marker of necrosis, in the poly I:C-stimulated MCD group compared to controls.

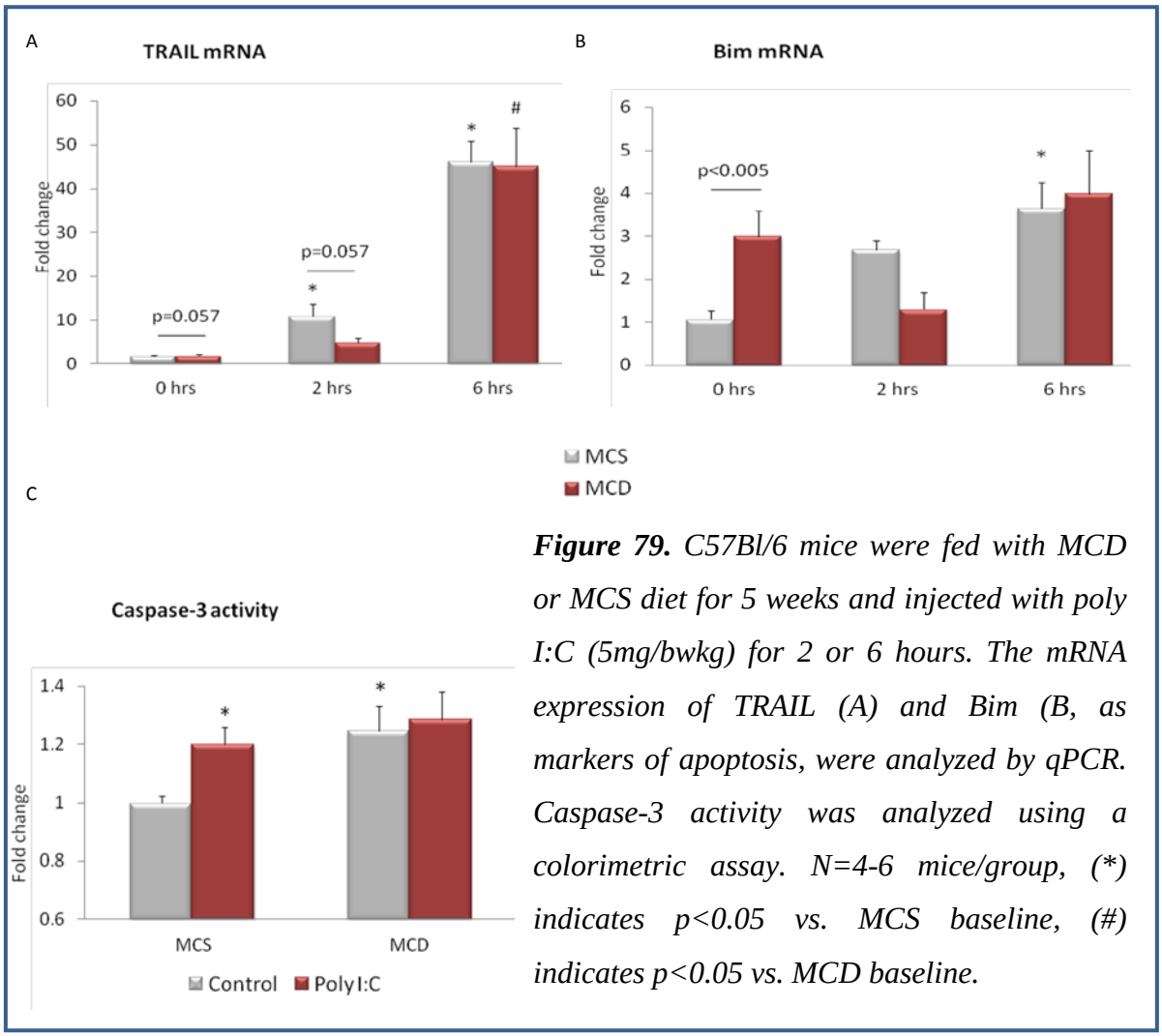

Figure 79. *C57Bl/6 mice were fed with MCD or MCS diet for 5 weeks and injected with poly I:C (5mg/bwkg) for 2 or 6 hours. The mRNA expression of TRAIL (A) and Bim (B, as markers of apoptosis, were analyzed by qPCR. Caspase-3 activity was analyzed using a colorimetric assay. N=4-6 mice/group, (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

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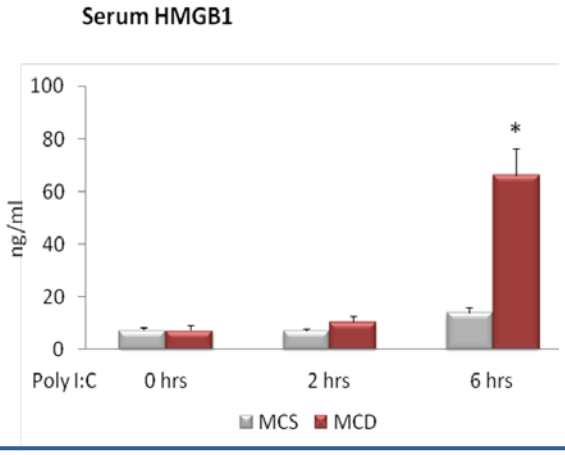
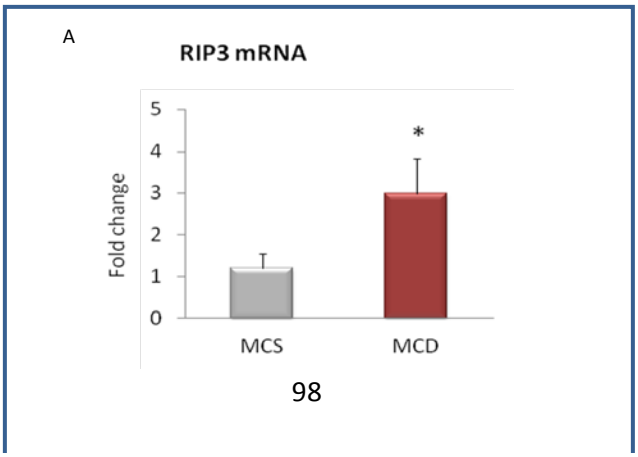


Figure 80. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and injected with poly I:C (5mg/bwkg) for 2 or 6 hours. Serum HMGB1 levels were measured by ELISA. N=4-6 mice/group, (*) indicates p<0.05 vs. MCS baseline.*

The balance between apoptosis and necrosis is tightly regulated (212). A recently identified master regulator between apoptosis and necrosis is the protein kinase receptor-interacting protein 3 (RIP3) (212). We found increased levels of RIP3 mRNA *(Figure 81A)* and protein *(Figure 81B)* in livers of MCD- compared to MCS-diet-fed controls. In control mice poly I:C stimulation induced upregulation of RIP3 protein expression at 2 hours post-stimulation which returned to baseline by 6 hours *(Figure 81B)*; in contrast, there was sustained induction of RIP3 in steatohepatitis after poly I:C challenge *(Figure 81B)*. We further identified a positive correlation between RIP3 and liver HMGB1 *(Figure 81C)* expression. Collectively, these data suggested that pathways that promote necrosis are preferentially upregulated in steatohepatitis after a viral challenge, at least in part due to the regulatory involvement of RIP3.



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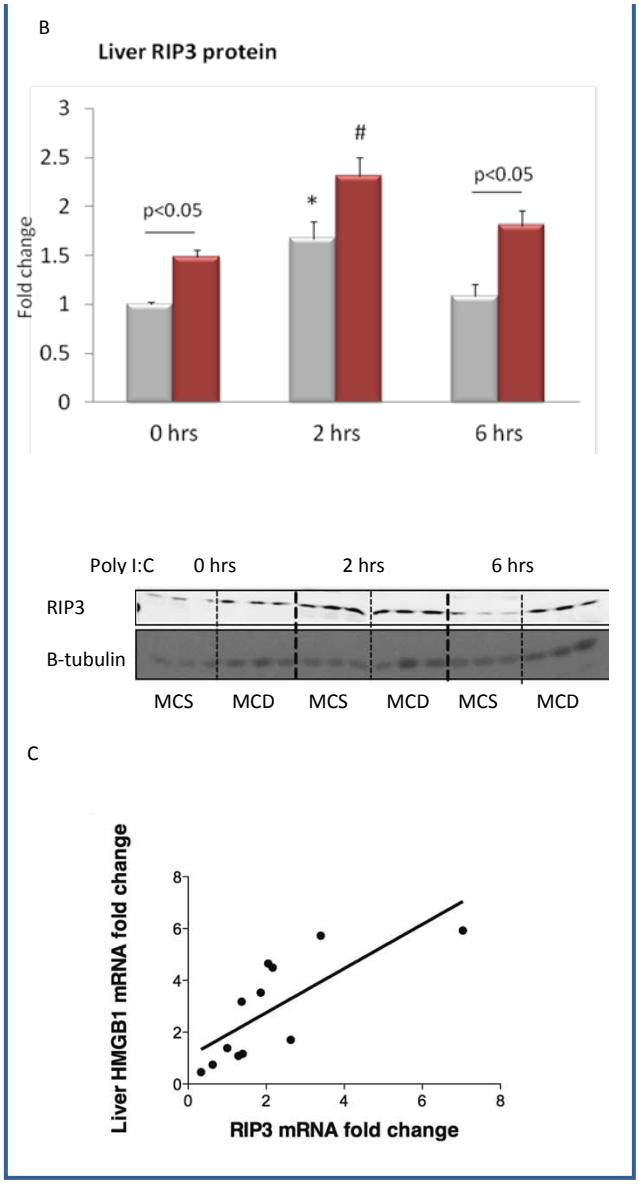

Figure 81. *C57Bl/6 mice were fed with methionine-choline-deficient (MCD) or –supplemented (MCS) diet for 5 weeks and injected with poly I:C (5mg/bwkg) for 2 or 6 hours. Liver mRNA (A) and protein (B) levels of RIP3 were analyzed in whole liver. Correlation between RIP3 and HMGB1 mRNA in the liver is shown (C). (*) indicates p<0.05 vs. MCS baseline, (#) indicates p<0.05 vs. MCD baseline.*

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5.3.7 Altered MAVS and RIP3 mRNA expression in human NASH

To validate our observations in the mouse model of steatohepatitis, we next evaluated human livers. We found an increase of MAVS mRNA levels in livers of NASH patients compared to healthy controls *(Figure 82A)* and this mirrored MAVS RNA levels in the animal model of steatohepatitis *(Figure 66).* MAVS mRNA up-regulation was specific to NASH since we did not observe increased MAVS levels in HBV-infection (HBV is a DNA virus) or in liver tumors (no viral infection detected) *(Figure 82A).* We also found higher expression of PSMA7 mRNA in human NASH livers *(Figure 82B)* that mirrored findings in the mouse model *(Figure 69).* Finally, we detected highly increased RIP3 mRNA levels in NASH patients *(Figure 84C)* compared to controls; this was parallel to the RIP3 mRNA increase in the mouse model of NASH *(Figure 82).*

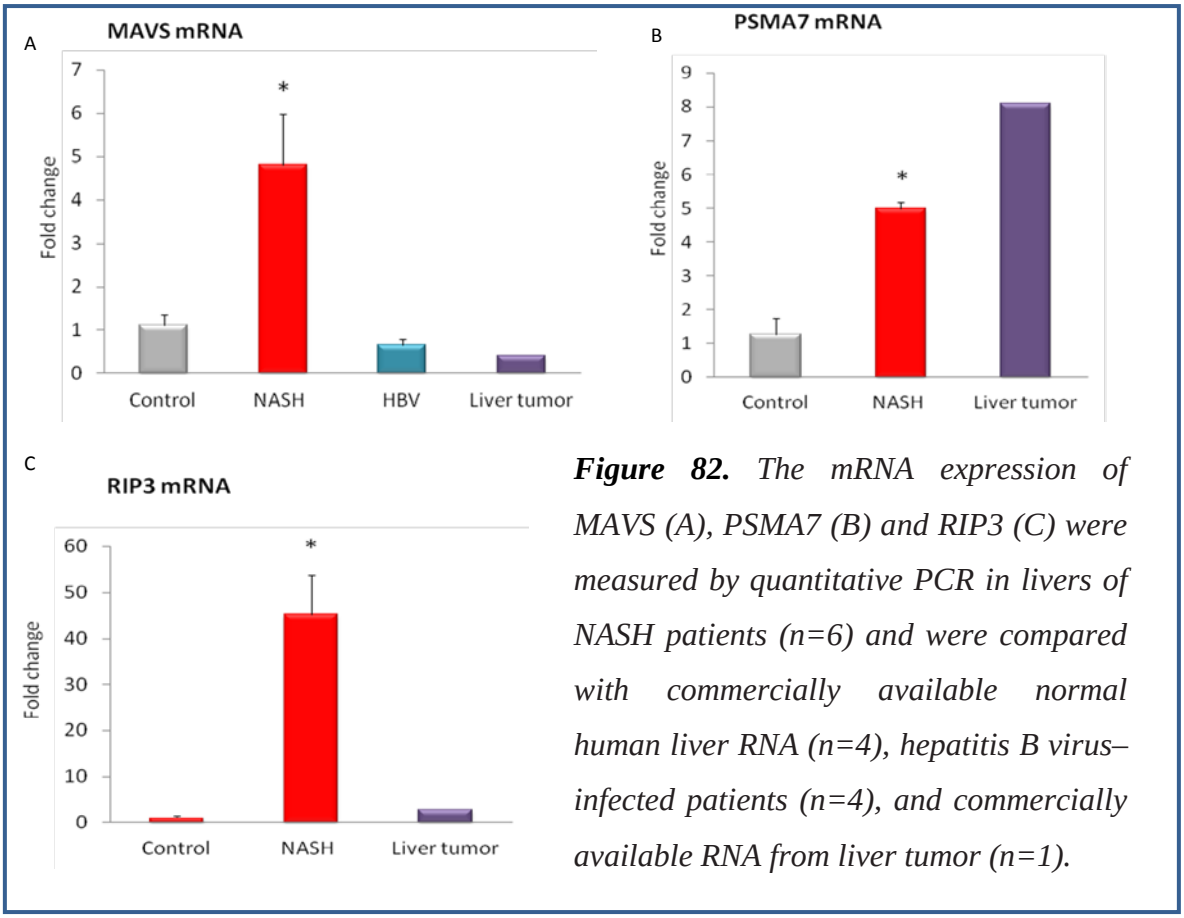

Figure 82. *The mRNA expression of MAVS (A), PSMA7 (B) and RIP3 (C) were measured by quantitative PCR in livers of NASH patients (n=6) and were compared with commercially available normal human liver RNA (n=4), hepatitis B virus–infected patients (n=4), and commercially available RNA from liver tumor (n=1).*

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6. DISCUSSION

In the present study we demonstrate several novel findings that supports the substantial role of innate immunity in the pathogenesis of NASH.

I. We demonstrate for the first time that deficient integrity of the danger receptor complex, including TLR4 or its co-receptor MD-2, is protective from MCD-diet-induced liver steatosis and inflammation, and correlates with attenuated liver injury and histological features of NASH. To this extent, our novel data also indicate that the deficiency in MD-2 or TLR4 confers protection from development of liver fibrosis in MCD-diet-induced NASH.

To date, several research groups have identified that LPS, in the context of a multi-hit model, plays a role in development of NAFLD/NASH (44,79-84); the details of LPS implication *per se* are yet to be fully defined. Here we provide novel data indicating that danger sensing via MD-2 and TLR4 is key in the pathogenesis of NASH. Ligand recognition by the TLR4/MD-2 complex, which binds LPS to deliver intracellular signals, occurs as a result of complementary functions of MD-2 and TLR4. Neither MD-2 nor TLR4 alone can account for optimal LPS recognition (213,214,215). MD-2 binds LPS however it lacks a transmembrane domain and cannot result in intracellular signaling alone (213-215). The recently discovered crystal structure of the TLR4/MD-2 complex demonstrates the critical role of MD-2 in LPS binding and LPS-induced TLR4 activation resulting in TLR4/MD-2 complex and conformational changes to initiate intracellular signaling through the intracellular domain of TLR4 (216). Our data suggest a major role for TLR4 and MD-2 in liver damage, as indicated by profound attenuation of features of NASH in their absence. The exact ligand(s) of TLR4/MD-2 in NASH is yet to be defined. A candidate ligand is endotoxin, most likely derived from the gut (82). This hypothesis is supported by recent reports in other models of non-alcoholic fatty liver disease and is also consistent with the causal role of gut-derived endotoxin in alcoholic steatohepatitis, which shares many pathological features of NASH (217,218,219). We found moderate but

significant increase in serum endotoxin levels in MCD-diet-fed mice of control genotypes; this observation is similar to that described in the portal circulation of LPS-insensitive C3H/HeJ mice (82).

In evaluation of the role of TLR4/MD-2 complex in the pathogenesis of NASH there is a need to consider that while TLR4 recognizes exogenous danger signals, such as LPS, it also can sense multiple endogenous danger signals (220), including, but possibly not limited to, heat-shock proteins (221), fibrinogen (222), fibronectin (223), and HMGB1 (224). Our results suggest protection from murine NASH when the recognition of ligands by TLR4/MD-2 complex is impaired; the role of endogenous danger signals in experimental or human NASH is yet to be evaluated.

We identified that MD2 and TLR4 deficiency is protective in NASH due to interference with inflammation and oxidative stress. The elements of protections included prevention of inflammatory cell infiltration into the liver, diminished pro-inflammatory cytokine production, impaired up-regulation of the liver mRNA levels of all components of the NADPH oxidase complex and impaired function of the NADPH complex. Our observation of increased expression of the phagocyte-specific NADPH complex and increased NADPH activity in MCD-fed animals of control genotypes and lack of such effects in TLR4 or MD-2 KO animals suggests a communication between TLR4/MD-2 and NADPH oxidase activation in NASH. Several research groups have reported the key role of the pro-inflammatory effects of Kupffer cells (82) and TLR4 receptor (44,82) in NASH-associated liver inflammation; our data are in agreement with those reports. Kupffer cells are rich in TLR4/MD-2 receptor complex (225), and are a major source of NADPH in the liver. The critical role of the Kupffer cells p47 phox NADPH oxidase component has been reported in alcoholic liver disease (226).

The most important clinical challenge in NASH is the progression to liver fibrosis, which often leads to cirrhosis and liver failure (1). Here, we present our novel observation that MD-2/TLR4 complex plays a central role in induction of fibrosis in the MCD-diet-induced NASH model. The current understanding on the pathogenic mechanisms of NASH favors a model in which steatosis, and later steatohepatitis, are induced as a result of fatty acid overload and inflammation, leading to subsequent activation of stellate cells resulting

in liver fibrosis (227,228,229). The critical step in the generation of liver fibrosis is the activation of stellate cells resulting in a-SMA and collagen deposition (227-229). Stellate cell activation is induced by multiple insults, including endotoxin, TNFα, and TGFβ (227,228). Stellate cells express MD-2 and TLR4, and thus, can be directly stimulated through the TLR4/MD-2 complex to produce a pro-fibrotic transformation including expression of α-SMA and deposition of collagen-1 (101,225,227). We found increased expression of α-SMA and collagen-1 at the mRNA and protein levels in livers of MCD-diet-fed animals of control genotypes that was abrogated in both MD-2-deficient and in TLR4-deficient mice. A recent finding that TLR4/MD-2 fusion protein could prevent LPS-induced stellate cell activation highlights the importance of the TLR4/MD-2-dependent mechanisms of stellate cell activation (230), suggesting that inhibition of TLR4/MD-2 is a potential therapeutic target. It remains to be determined whether the protective effect of deficiency of MD-2 and TLR4 on fibrosis is solely related to the lack of TLR4/MD-2 activation in stellate cells or a combined lack of TLR4 signaling in stellate and Kupffer cells. We identified increased TNFα, a product of activated Kupffer cells, in MCD-diet-induced NASH, which can induce stellate cell activation (228). Importantly, TNFα levels were significantly attenuated in the absence of MD-2 or TLR4 expression. NADPH oxidase dependent oxidative stress has been reported to have crucial role in hepatic stellate cell activation by angiotensin II and leptin (231,232). Although, TLR4/MD-2 deficiency attenuated both the fibrosis and the NADPH oxidase activity, further experiments are needed to prove causality between NADPH oxidase activity and fibrosis in NASH. However, our novel data and the availability of relatively safe and well-established therapeutics to manipulate the NADPH oxidase-dependent oxidative stress brings hope for future NADPH-based therapeutic interventions in steatohepatitis (233).

Our study is based on 2 distinct models of genetically-modified mice: TLR4-KO and MD-2-KO. While both types of animals exhibit impaired recognition of LPS due to deficient assembly of the recognition complex, we did observed some subtle differences between TLR4 KO and MD-2 KO mice upon developing MCD diet-induced NASH. For example, the extent of liver fibrosis, indicated by the Sirius red positive areas, was more pronounced in MD-2 KO compared to TLR4 KO etc. The origin of the lack of full overlap

between the TLR4- and MD-2-owed extent of protection against NASH likely lies in the final effect of these molecules on ligand recognition and/or downstream signaling events. MD-2 is an important component of LPS recognition, however it may, or may not, be implicated in the recognition of the entire repertoire of TLR4 ligands. Alternatively, TLR4 with or without MD-2 may signal differently, or TLR4-MD-2 complex receptor may function in two separate modes: one in which full signaling occurs and one limited to MyD88-dependent signaling (234). We had previously reported a critical role of toll-like receptors and the common TLR adaptor, MyD88, in other models of liver inflammation and injury (235); the exact signaling events downstream from TLR4-MD-2 complex in NASH are yet to be fully understood. Nevertheless, it is important to note that both TLR4 KO and MD-2 KO genotypes offered only partial protection against MCD diet-induced NASH, suggesting the possibility that TLR4/MD-2-independent events may be involved in the pathogenesis of NASH.

In conclusion, we found that danger receptor TLR4 and its co-receptor, MD-2, are critical in the development of steatosis, liver damage, inflammation and fibrosis in the MCD-diet-induced NASH in mice.

Given that the TLR4/MD2 complex is the major receptor for LPS that is also a key factor of the inflammasome activation and IL-1β production, in the second part of the study we investigated the role of inflammasomes in the pathogenesis of NASH.

Inflammation is a response to cellular injury or pathogens and it is triggered by endogenous and exogenous danger signals, respectively. NALPs, the receptor components of the inflammasome, sense endogenous danger signals which activate the inflammasome, a multiprotein complex involved in caspase-1-mediated IL-1 cleavage. Inflammasome activation is typically a result of a two signals via TLR activation by exogenous or endogenous danger signals (116).

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II. Here we report several findings related to the novel role of inflammasome activation in non-alcoholic steatohepatitis. First, we show upregulation of the components of the NALP3 inflammasome, including, NALP3, ASC and pro-caspase-1 in NASH in mouse models as well as in human livers, and demonstrate functional activation via caspase-1 activation and IL-1ß production. Our data also suggest that inflammasome activation occurs in steatohepatitis and not in early steatosis in mice. Second, we report that while increased circulating endotoxin likely contributes to inflammasome activation, exogenous LPS can amplify inflammasome activation and IL-1ß secretion in steatohepatitis. Third, we demonstrate for the first time that inflammasome activation and IL-1ß secretion occur in isolated hepatocytes in NASH. Fourth, we reveal a mechanistic insight into inflammasome activation and show that saturated, but not un-saturated, fatty acids increase inflammasome expression and sensitize hepatocytes to IL-1ß release by a second stimulus via TLR4 activation. Fifth, our novel data show that fatty acids not only upregulate inflammasome but also induce apoptosis and release of danger signals in hepatocytes. We report for the first time that danger signals from fatty acid-exposed hepatocytes induce inflammasome activation in liver mononuclear cells demonstrating a cross-talk between injured hepatocytes and inflammatory cells in NASH. Finally, we show that IL-1 signaling contribute to the development of liver steatosis; however, neither the lack of the inflammasome effector caspase-1, nor IL-1R deficiency prevents liver injury and/or fibrosis in MCD diet-induced steatohepatitis.

Both NALP3 and NALP1 are highly expressed in primary immune cells, but also in other cell types, including epithelial cells, neurons, and gonadal cells (152). Here we report that hepatocytes express NALPs. We identified that hepatocytes express the adaptor molecule ASC and the entire functional inflammasome machinery and capable of IL-1ß production.

Elucidation of the triggering factors responsible for the increased inflammasome expression and function in NASH is of emerging importance. Free fatty acids can be recognized as endogenous danger molecules and induce inflammatory signaling and activation of NFκB and JNK-AP1 pathways leading to cytokine and chemokine production

(236,237). While toll like receptors (TLRs) detect ligands either on the cell surface or in the lumen of endoplasmatic reticulum (220), Nod-like receptors (NLRs) are intracellular, cytoplasmic (NALP3) or nuclear (NALP1) sensors (152). We found that saturated fatty acids induce up-regulation of pro-IL1β and NALP3 in hepatocytes. Increased FFA levels have been reported in MCD diet- (238), HFD- (239) and leptin deficiency induced (240) steatohepatitis as well as in human NAFLD patients with either steatosis or steatohepatitis (241,242). While several reports evaluated the fatty acid profile and the ratio of saturated and unsaturated fatty acids in animal models (238-240) and in human plasma in NASH (241,242), it is yet to be determined whether changes in fatty acid composition in the liver or serum correlate with steatosis or steatohepatitis. We speculate that saturated fatty acids in NASH may favor inflammasome activation while a different composition of FFA in simple steatosis may not trigger such events. These differences could be further amplified by the presence of additional signals such as LPS or danger signals from damaged hepatocytes.

There is accumulating evidence that innate immune pathways are activated in the metabolic syndrome and play a crucial role in the pathogenesis of NASH (65). Increased plasma levels of the Toll-like receptor-4 (TLR4) ligand, LPS, and enhanced susceptibility to LPS-induced liver damage have been observed (44,80,81). We found increased serum endotoxin levels in mice with steatohepatitis suggesting the presence of an exogenous TLR ligand. We, and others, have shown that TLR4 deficiency can prevent experimental NASH (44,82). Exogenous administration of LPS further increased IL-1ß levels and inflammasome expression in livers with steatohepatitis suggesting that the fatty liver is primed for LPS-induced inflammasome activation. This novel observation complements previous reports that demonstrated that the fatty liver is sensitized to LPS-induced TNFα production and LPS-induced liver damage (44). TLR4 deficiency and modulation of TLR4 pathways with probiotics that alter intestinal flora and suppress TLR-related responses, improved liver injury and inflammation in NASH (44,82). More importantly, we identified an increased inflammasome function, indicated by cleavage of pro-caspase 1 and increased IL-1 production, along with the increased expression of inflammasome in our NASH model.

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We also demonstrate that saturated fatty acids contribute to the sensitization of LPS-induced IL-1β secretion in hepatocytes. It remains to be further examined whether the effect of fatty acids on inflammasomes is direct or indirect, through intermediate products of FFA metabolism or via FFA-induced cell death (243) and release of DAMP molecules. However, our finding that pan-caspase inhibitor ZVAD can prevent the FFA-induced inflammasome up-regulation suggests a role of lipotoxicity and endogenous danger molecules in this process (244,245). Saturated fatty acids (eg. palmitic) are more toxic and apoptotic, while monounsaturated fatty acids (eg. oleic acid) are lipogenic and protect from the apoptotic effect of saturated FAs in cell cultures (246). Palmitic acid + LPS together lead to inflammasome and caspase-1 activation. In contrast, palmitic acid alone induced only caspase-8 activation without detectable inflammasome activation, suggesting that caspase-8 is responsible for the IL-1β cleavage in PA-treated hepatocytes. Caspase-8 has been shown as an alternate to cleave pro-IL-1β in macrophages in response to TLR3 and TLR4 stimulation (193). A caspase-1-independent IL-1β release was also reported in apoptosis induced by Fas ligand in peritoneal immune cells (247). Here we demonstrate that danger signals released from damaged hepatocytes upon saturated FA treatment trigger inflammasome activation in liver mononuclear cells.

Previous studies showed enhanced inflammatory response and liver injury to LPS in NASH (44). It is likely that in addition to gut-derived LPS other danger signals from hepatocytes are also increased. It was found that a brief pre-stimulation with ATP leads to robust LPS-induced caspase-1 activation and IL-1beta secretion in macrophages (248). Our data suggest that a sensitization to LPS-induced inflammasome activation and IL-1β secretion occurs in the fatty liver; IL-1β then can further amplify the inflammatory response through IL-1-receptor. Finally, we could not exclude that besides fatty acids, alternative activators of the inflammasome, such as ATP, monosodium urate crystals (MSU), or calcium pyrophosphate, may contribute to inflammasome activation in the fatty liver.

To test the physiological significance of the above described inflammasome activation in NASH, we employed mice deficient in either the inflammasome adaptor ASC or the effector molecule caspase-1. We showed the first time that although, steatohepatitis

is accompanied with inflammasome activation; the lack of neither ASC nor caspase-1 was protective in MCD-diet-induced steatohepatitis. It is in accordance with the observation that the administration of pan-caspase inhibitor attenuated hepatic steatosis and fibrosis in db/db+MCD-diet model of steatohepatitis in mice, but did not affect liver injury (245). *Vandanmagsar et al.* reported that NLRP3 deficient mice exhibit diminished fat accumulation in the liver upon long term high fat diet feeding (249), however, there are no reports on liver injury or fibrosis.

Interestingly, the caspase-1 KO mice showed increased levels of IL-1β in the MCD-fed mice. Caspase-1 is one of the proteases that process pro-IL-1β but not the only one, as we mentioned earlier. Caspase-8 has been shown as an alternate to cleave pro-IL-1beta in macrophages in response to TLR3 and TLR4 stimulation (193). Here, we found increased caspase-8 activity in livers with steatohepatitis both in wild type and caspase-1 knock out mice, which arose that IL-1β cleavage may happen by caspase-8 in NASH. Furthermore, our data suggested that caspase-8 derived processing of IL-1β could compensate the lack of caspase-1 and result in the production of enough biologically active IL-1β to maintain the inflammation. Recently, neutrophil-derived serine proteases have been also suggested to be able to cleave pro-IL-1β besides caspase-8 (250). Enhanced neutrophil infiltration is known in NASH (1,24), however the role in processing IL-1β remains out of focus of present investigation. Altogether, our data suggested that caspase-1 is not the only enzyme that is responsible for the IL-1β maturation in our model.

To further investigate the relevance of IL-1β in MCD-diet-induced steatohepatitis, we employed IL-1R KO mice. Similarly to previous reports (171), we found attenuated hepatic steatosis in IL-1R KO mice after MCD-diet feeding. However, in contrary to the findings in CDAA model (171), we found no protection against liver injury or fibrosis. This suggested that while IL-1β contributes to liver steatosis, it has no substantial effect on liver injury in every model of steatohepatitis that makes difficult the translation to human disease.

In summary, we propose that the increased saturated fatty acid influx to the liver leads to inflammasome activation, IL-1β cleavage and inflammation. We also show that saturated FA induces hepatocyte apoptosis and activation of caspase-8 that triggers the release of danger molecules. All together, these events synergize with circulating endotoxins to result in inflammasome activation in the hepatocytes and might create an amplification loop of inflammation by activating liver mononuclear cells and inducing liver injury. However, we also showed that IL-1β production in NASH involves both classical (inflammasome-dependent) and alternative (caspase-8 dependent) pathways. Finally, our novel data demonstrate that while IL-1β is important player in the development of liver steatosis, it is not the only or key factor of the development of liver injury in MCD diet-induced animal model of steatohepatitis.

Not only the factors determining progression of NASH are yet to be fully defined, but we also have to mention that steatosis and steatohepatitis are co-factors in the progression of other liver diseases, including those of viral etiology, ischemia-reperfusion injury and liver transplantation (42-46).

III. Here we report novel findings related to the impaired capacity of the fatty liver to respond to dsRNA and related viral challenges: First, livers with steatohepatitis failed to activate anti-viral innate immune pathways to produce Type I IFNs in response to a double-stranded RNA challenge. Second, the MAVS adapter, which is required for Type-I IFN induction after recognition of dsRNA by the helicase receptors RIG-I and Mda5, was dissociated from the mitochondria to the cytosol and showed impaired oligomerization and function in steatohepatitis. Third, displacement of MAVS from mitochondria was associated with oxidative stress and instead of upregulation of the apoptosis cascade, poly I:C promoted necrosis via increased expression of RIP3 in steatohepatitis. Fourth, dsRNA challenge resulted in increased liver damage in spite of decreased TNFα and pro-inflammatory cytokine induction in a diet-induced model of NASH.

Viral-sensing receptors include toll-like receptor (TLR) 3 and the cytoplasmic helicase receptors RIG-I and Mda5 for dsRNA recognition, TLR7/8 for ssRNA and TLR9 for sensing viral DNA (102). Here we identified a selective defect in signaling from viral

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dsRNA in steatohepatitis that altered both of pro-inflammatory cytokines and Type-I IFNs and was associated with increased liver damage. Although TLR3, Mda5 and RIG-I all sense poly I:C, their signaling pathways are different. Mda5 plays a key role in poly I:C-induced IFNβ production even in the absence of TLR3 or RIG-I (102). Ligand engagement of the helicase receptors catalyzes the phosphorylation of IκB proteins by IKK complex and leads to NFκB activation, along with the phosphorylation and activation of IRF3 (102). NFκB activation triggers the production of pro-inflammatory cytokines, while IRF3 phosphorylation leads to production of Type-I interferons (102).

The cellular source of the Type-I IFNs and inflammatory cytokines remains to be evaluated. Helicase receptors are expressed in several cell types in the liver, including hepatocytes, conventional dendritic cells, Kupffer-cells, NK cells (251,110). RLR expression is enhanced by poly I:C (250). We found that hepatocytes that represent the majority of cells in the liver produce IFNβ after intracellular poly I:C stimulation in vitro (data not shown). The RIG-I/Mda5 pathway is also important in the conventional dendritic cells (249) and NK cells (252), but less prominent in plasmacytoid dendritic cells. Thus, we speculate that hepatocytes and conventional DCs are the likely sources of Type-I IFN production after dsRNA challenge in the liver. Previous studies demonstrated a role of NK cells in NASH (210). Here we found evidence for increased expression of NK cell activating ligands, PanRae, Rae1α, Mult-1 in livers with steatohepatitis without a further increase after dsRNA stimulation. We also determined that NK cell recruitment was not triggered in livers with NASH suggesting that the liver damage was unlikely to be NK cell mediated after poly I:C challenge.

Here we demonstrated that both Type-I IFNs and pro-inflammatory cytokine induction were selectively disturbed in response to dsRNA while TLR4- or TLR9-mediated pathways remained intact in steatohepatitis. This suggested that the signaling defects in fatty livers occurred upstream from the branching of the NFκB and IRF3 signaling pathways and involved a protein that is common to both pathways upon dsRNA stimulation. The mitochondrial antiviral signaling protein (MAVS) mediates the activation of both NFκB and IRF3 in response to viral infection (105). Here we show for the first time that total liver MAVS protein levels are decreased in steatohepatitis. Our data showed

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increased association of MAVS with the proteasome subunit PSMA7 in MCD-steatohepatitis suggesting that proteosomal degradation could contribute to low MAVS levels. In this context, the apparent discrepancy between our finding of decreased MAVS protein and increased liver MAVS RNA could represent a compensatory feed-back loop mechanism. Increased mRNA levels of MAVS and PSMA7 were also present in human livers with NASH.

Impaired MAVS function was suggested by three of our novel observations. First, MAVS levels were decreased in the mitochondria with a complementary increase in the cytosol in the mouse model of steatohepatitis compared to controls. Second, in parallel with the MAVS dissociation from the mitochondria, we found decreased MAVS oligomerization in livers of MCD diet-fed mice compared to controls. Third, we found impaired induction of IRF3 phosphorylation by poly I:C in livers with steatohepatitis.

The transmembrane domain (TM) of MAVS is crucial to the mitochondrial localization of MAVS, but also required for the dimerization of the protein that is a crucial step during MAVS induced immune responses. (106,107) Our novel finding on the reduced MAVS oligomerization is in accordance with the impaired function of the helicase receptor-MAVS signaling pathway.

Mitochondrial dysfunction is a key component of fat accumulation, ROS generation and the progression of inflammation in NASH (88). Thus, it is plausible that translocation of MAVS from the mitochondria to the cytosol could be a consequence of mitochondrial damage in steatohepatitis. In addition to MAVS redistribution, we found other indications of mitochondrial damage, such as cytochrome C leak from the mitochondria to cytoplasm, enrichment of mitochondria with β-actin, and increased activation of cellular damage pathways. Translocation of β-actin to the mitochondria leading to disruption of mitochondrial membrane was shown in influenza virus-stimulated macrophages (253). We found markedly elevated β-actin protein levels in mitochondrial fractions in steatohepatitis providing evidence for mitochondrial damage in NASH. In normal hepatocytes, MAVS is localized in the outer mitochondrial membrane (106). Our novel data indicate increased activation of multiple caspases, including caspase 1 and caspase-8, in MCD diet-induced steatohepatitis suggesting a possible link between MAVS

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cleavage and caspase activation. Several viruses, including hepatitis C (NS3/4A protease) and hepatitis A (3ABC protease), disrupt the host antiviral response by cleaving MAVS from mitochondria (206,207). An apoptotic cleavage of MAVS has also been described (207). In NASH, both the death-receptor induced, and cellular-stress induced, apoptotic pathways are involved and apoptosis is indicated by increased caspase-3 activity and plasma cytokeratin-18 fragments (12,254). Studies showed that the pancaspase inhibitor, zVAD, prevents the cleavage of MAVS, while selective blockade of caspase-8, -9 or -3 was not sufficient to prevent MAVS cleavage (207). Relevant to our data, the pan-caspase inhibitor blocks both apoptotic caspases and caspase-1 (207,208). Thus, MAVS cleavage from the mitochondria in NASH is likely to be related to the increased caspase-8 and caspase-1 observed in our experiments.

Damaged proteins are degraded by proteasomes in the cytoplasm or nucleus (255). We show here first time that MAVS protein preferentially binds to the proteasomal protein PSMA7 in fatty livers, suggesting that the damaged, cleaved MAVS protein from the mitochondria accumulates in the cytoplasm, and likely degraded by the proteasomes.

Virus-induced apoptosis requires MAVS in primary mouse fibroblasts (211) and MAVS itself can induce caspase-dependent apoptosis. It has been shown that poly I:C initiates apoptosis via MAVS (256). However, MAVS levels were decreased in MCD diet-induced steatohepatitis in our experiments. Furthermore, we found that while caspase-3 was activated by dsRNA stimulation in normal liver suggesting apoptosis, there was no increase over the elevated baseline apoptosis (caspase-3 activity) in steatohepatitis. Instead, poly I:C induced liver necrosis and increased serum HMGB1 levels in MCD diet-fed mice. We speculate that decreased mitochondrial MAVS levels may result in impaired MAVS-dependent apoptosis following dsRNA challenge in MCD-steatohepatitis.

MAVS interacts with protein kinase receptor-interacting protein 1 (RIP1) and facilitates NFκB activation (257). RIP1 and the protein kinase receptor-interacting protein 3 (RIP3) may form a complex with TRADD, FADD and caspase-8 that leads to RIP3 cleavage and proteolytic inactivation (258,259). Studies showed that RIP3 overexpression results in TNFα and NO-mediated necrosis (257,260). RIP3 has been identified as a molecular switch between apoptosis and necrosis (261). Here we show for the first time

that increased expression of RIP3 in MCD-diet fed mice occurs both at the mRNA and protein levels. Increased RIP3 mRNA was also present in human livers with NASH. We found a sustained increase in RIP3 expression that correlated with increased necrosis and increased serum HMGB1 levels after poly I:C challenge in steatohepatitis in mice. It is tempting to speculate that increased RIP3 may result in an apoptosis-to-necrosis switch after a dsRNA challenge in steatohepatitis. Recent studies suggest RIP3 association with the mitochondria and its regulation by ROS (257) and RIP3-induced promotion of necrosis is regulated by ROS (259). Our observations confirmed previous findings of increased ROS generation in diet-induced NASH (88). More importantly, we identified that poly I:C augmented ROS generation as well as RIP3 induction and necrosis in MCD-induced steatohepatitis.

In conclusion, our data demonstrate an important role for mitochondrial damage and MAVS dissociation from the mitochondria in the increased susceptibility of steatohepatitis to a dsRNA viral challenge. We report for the first time that livers with steatohepatitis fail to induce Type-I IFNs in response to dsRNA challenge due to dissociation of MAVS from the mitochondria and impaired oligomerization. The MAVS dissociation also leads to impaired induction of apoptosis and promote necrosis together with increased RIP3 expression, impaired anti-viral interferon response and increased liver damage in non-alcoholic steatohepatitis. These key findings were also reproducible in human NASH.

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7. CONCLUSIONS

In the present study we demonstrate several novel findings that support the substantial role of innate immunity in the pathogenesis of NASH.

The following conclusions can be drawn based on our results:

1. Deficient integrity of the danger receptor complex, including TLR4 or its co-receptor MD-2, is protective from MCD-diet-induced liver steatosis, inflammation, correlates with attenuated liver injury and confers protection from development of liver fibrosis in MCD-diet-induced NASH.
2. There is up-regulation and activation of the NALP3 inflammasome complex and therefore increased IL-1β production in NASH in mouse models as well as in human livers. Our data also suggest that inflammasome activation occurs in steatohepatitis and not in early steatosis in mice.
3. While increased circulating endotoxin likely contributes to inflammasome activation, exogenous LPS can amplify inflammasome activation and IL-1ß secretion in steatohepatitis.
4. Inflammasome activation and IL-1ß secretion occur in isolated hepatocytes in NASH.
5. Saturated, but not un-saturated, fatty acids increase inflammasome expression and sensitize hepatocytes to IL-1ß release by a second stimulus via TLR4 activation.
6. Fatty acids not only upregulate inflammasome but also induce apoptosis and release of danger signals in hepatocytes.
7. These danger signals induce inflammasome activation in liver mononuclear cells demonstrating a cross-talk between injured hepatocytes and inflammatory cells in NASH.
8. The IL-1 signaling contributes to the development of liver steatosis; however, neither the lack of the inflammasome effector caspase-1, nor IL-1R deficiency prevents liver injury and/or fibrosis in MCD diet-induced steatohepatitis.
9. There is increased inflammasome expression in the liver of NASH patients.

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Finally, here we report novel findings related to the impaired capacity of the fatty liver to respond to dsRNA and related viral challenges:

10. Livers with steatohepatitis fail to activate anti-viral innate immune pathways to produce Type I IFNs in response to a double-stranded RNA challenge.
11. The MAVS adapter, which is required for Type-I IFN induction after recognition of dsRNA by the helicase receptors RIG-I and Mda5, is dissociated from the mitochondria to the cytosol and shows impaired oligomerization and function in steatohepatitis.
12. The displacement of MAVS from mitochondria is associated with oxidative stress and instead of upregulation of the apoptosis cascade, poly I:C promotes necrosis via increased expression of RIP3 in steatohepatitis.
13. Fourth, we show that dsRNA challenge results in increased liver damage in spite of decreased TNFα and pro-inflammatory cytokine induction in a diet-induced model of NASH.

Our novel data might contribute to better understand the pathogenesis of non alcoholic steatohepatitis and therefore to develop targeted therapy.

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8. SUMMARY

The pathogenesis of non-alcoholic steatohepatitis (NASH), one of the most common liver diseases, is not fully understood yet. Recently the role of innate immunity has been implicated in the pathogenesis of NASH. The aim of thesis was to explore and contribute to the better understanding of the role of innate immunity in the pathogenesis of NASH.

In the first part of the work we focused on the role of the Gram negative bacterial wall component endotoxin and its receptor, Toll-like receptor 4 in the development of diet-induced steatohepatitis and fibrosis. We showed that deficiency TLR4-MD-2 receptor complex is protective from MCD-diet-induced liver steatosis, inflammation, correlates with attenuated liver injury and confers protection from development of liver fibrosis in MCD-diet-induced NASH.

In the second part of the work we investigated the role of the pro-inflammatory cytokine IL-1β and the inflammasome complexes that are responsible for the IL-1β-maturation in the pathogenesis of NASH. We showed that there is inflammasome activation and increased IL-1β production in various animal models of NASH. We proposed that the increased saturated fatty acid influx to the liver together with the gut-derived endotoxin contributed to the inflammasome activation. Furthermore, we showed that there is a crosstalk between hepatocytes and immune cells. However, our data also demonstrated that while IL-1β is important player in the development of liver steatosis, it is not the only or key factor of the development of liver injury in MCD diet-induced animal model of steatohepatitis.

In the third part of the work we aimed to explore the pathogenesis behind the susceptibility of fatty liver to viral diseases. Our data demonstrated an important role for mitochondrial damage and MAVS dissociation from the mitochondria in the increased susceptibility of steatohepatitis to a dsRNA viral challenge.

Our novel findings support the substantial role of innate immunity, including toll-like receptors (TLR4), inflammasome complexes (NALP3) and pro-inflammatory cytokines (TNFα and IL-1β) in the pathogenesis of NASH.

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9. ÖSSZEFOGLALÁS

Nem alkoholos steatohepatitis, ami az egyik leggyakoribb májbetegség, pathogenezise nem teljesen tisztázott. Az utóbbi években számos vizsgálat felvetette a természetes immunitás szerepét a NASH kialakulásában. Munkánk célja a természetes immunválasz szerepének vizsgálata volt a nem alkoholos zsírmáj pathogenezisében.

A munka első részében a Gram-negatív baktériumok fali alkotóelemének, az endotoxinnak illetve az endotoxin receptorának, a Toll-like receptor 4 (TLR4)-nek a NASH pathogenezisében betöltött szerepét vizsgáltuk. Eredményeink szerint a TLR4–MD2 receptor komplex hozzájárul a nem alkoholos zsírmáj pathogeneziséhez, érinti mind a zsír akkumuláció, gyulladás és fibrózis kialakulását lévén a TLR4-MD-2 knock-out egerek részlegesen védettek a diéta indukálta steatohepatitis kialakulása ellen.

A munka második részében egy gyulladásos citokin, az interleukin-1β, illetve az IL-1β aktiválásáért felelős intracelluláris multiprotein komplex, az inflammaszóma szerepét vizsgáltuk. Kimutattuk, hogy a nem alkohololos steatohepatitis az inflammaszóma aktiválódásával és ezáltal fokozott IL-1β termeléssel társul számos állatmodellben[1]. Eredményeink szerint a szaturált zsírsavak és a bakteriális eredetű endotoxin együttesen vezetnek az inflammaszóma aktivációjához. Továbbá kimutattuk, hogy az inflammaszóma komplex aktiválható izolált egér májsejtekben is és eredményeink alátámasztják a sérült hepatociták és immunsejtek közötti kommunikáció fontosságát. Mindazonáltal, míg a fokozott IL-1β termelés valószínűleg hozzájárul a steatosis kialakulásához, az általunk használt állatmodellben az IL-1 jelátvitel defektusa nem védte ki a májkárosodást és a NASH indukálta fibrózist

A munka harmadik részében a NASH betegekben megfigyelhető csökkent antivirális immunitás pathomechanizmusát vizsgáltuk. Eredményeink a mitokondriális károsodás ill. ennek következményeként a MAVS csökkent mitokondriális elhelyezkedésének fontosságára utaltak a csökkent antivirális immunitás hátterében.

A disszertációban részletezett munka számos új eredménnyel járult hozzá a nem alkoholos steatohepatitis pathogenezisének feltérképezéséhez és alátámasztja a természetes immunitás, a toll-like receptorok (TLR4), inflammaszóma komplex (NALP3) és gyulladásos citokinek (TNFα, IL-1β) fontos szerepét a NASH kialakulásában.

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10. PUBLICATIONS

Publications for the dissertation based on:

- **Csak T**#, Velayudham A#, Hritz I, Petrasek J, Levin I, Catalano D, Mandrekar P, Dolganiuc A, Kurt-Jones EA, Szabo G: Deficiency in myeloid differentiation factor-2 and Toll-like receptor 4 expression attenuates non-alcoholic steatohepatitis and fibrosis in mice. (# These authors contributed equally) *Am J Gastroenterol Physiol, Gastrointestinal and Liver Physiology, 2011;300:G433-41.* IF: 3.522
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