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ÚLOHA TOLL-LIKE RECEPTORŮ V PATOGENEZI JATERNÍCH ONEMOCNĚNÍ

THE ROLE OF TOLL-LIKE RECEPTORS IN THE PATHOGENESIS OF LIVER DISEASES

Disertační práce/Ph.D. thesis

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Abstrakt

Společným jmenovatelem nejčastějších onemocnění jater je aktivace mechanismů vrozené imunity, které přispívají k rozvoji zánětu a poškození jaterního parenchymu. Klíčovou úlohu v rozvoji jaterního poškození hrají Toll-like receptory, jejichž charakterizace v posledním desetiletí vedla přehodnocení patofyziologie některých jaterních onemocnění. Předkládaná práce studuje význam alelických variant v genech kódujících proteiny Toll-like receptorové signální kaskády a mezibuněčné signalizace v patogenezi alkoholické nemoci jater, přináší nový pohled na probiotika v léčbě nealkoholické steatohepatitidy a nové poznatky o protizánětlivém působení interferonů I. typu u některých jaterních chorob.

Abstract

Recent reports suggest that majority of chronic and acute liver diseases share a significant degree of liver inflammation and injury attributable to innate immunity, activated through Toll-like receptors. Detailed characterization of Toll-like receptor sigaling cascades in the last decade changed the view on the pathophysiology of liver injury by emphasizing the involvement of immune-mediated mechanisms. This thesis is focused on the role of allelic variants in genes encoding proteins of Toll-like receptor signaling pathways and cellular cross-talk in the pathogenesis of alcoholic liver disease, reports novel data on the role of probiotics in therapy of non-alcoholic steatohepatitis, and demonstrates anti-inflammatory role of Type I interferons in selected liver diseases, suggesting possible therapeutic implications.

Klíčová slova: Toll-like receptory, přirozená imunita, onemocnění jater

Keywords: Toll-like receptors, innate immunity, liver diseases

Table of contents

1. Introduction **14**

1.1 The role of Toll-like receptors in innate immunity **17**

1.1.1 Structure and ligands for cell surface Toll-like receptors 17

1.1.2 Structure and ligands for nucleic acid-sensing Toll-like receptors 18

1.1.2.1 Cellular localization of nucleic acid-sensing Toll-like receptors 20

1.1.2.2 Endogenous ligands for Toll-like receptors 20

1.1.3 Major intracellular pathways involved in Toll-like receptor signaling 21

1.1.3.1. The MyD88-dependent pathway in Toll-like receptor-induced signaling 24

1.1.3.2. The MyD88-independent/TRIF-dependent pathway in Toll-like receptor-induced signaling 25

1.1.4 Activation of the Interferon regulatory factors and Type I Interferons via the Toll-like receptor signaling 25

1.1.4.1 Interferon regulatory factor 3 (IRF3) and 7 (IRF7) 26

1.1.4.2 TLR7 and TLR9 signaling in plasmacytoid dendritic cells 27

1.1.4.3 The family of Interferons 29

1.1.4.3.1 Plasmacytoid dendritic cells as the major producers of Type I IFNs 29

1.1.4.3.2 Induction of Type I IFNs: a two-step hypothesis 30

1.1.4.3.3 Signaling triggered by Type I Interferons 31

1.2 Expression of Toll-like receptors in the liver **32**

1.3. Toll-like receptors in the pathogenesis of liver diseases **35**

1.3.1 Toll-like receptors in the pathogenesis of alcohol-induced liver injury 36

1.3.1.1 Gut-derived bacterial components are critical in the pathogenesis of ALD 37

1.3.1.2 Recognition of Toll-like receptor ligands in alcohol-induced liver injury 38

1.3.1.3 Downstream effects of TLR4 signaling in alcohol-induced liver injury 40

1.3.1.4 Transcription factors in alcohol-induced liver injury 40

1.3.1.5 Pro-inflammatory cytokine induction in ALD 41

1.3.1.6 Toll-like receptors and induction of oxidative stress in alcohol-mediated liver injury 42

1.3.1.7 Cell-specific effect of IRF3 in the pathogenesis of alcoholic liver disease 43

1.3.2 The role of genetic factors in the pathogenesis of alcoholic liver disease 44

1.3.2.1 Genetic susceptibility to alcoholic liver disease 44

1.3.2.2 Candidate pathways for genetic susceptibility to alcoholic liver disease 44

1.3.2.3 Genetic variability in genes modulating the TLR4-induced alcoholic liver injury 44

1.3.3 The role of probiotics in TLR4-mediated pathogenesis of non-alcoholic steatohepatitis 46

1.3.4 The Toll-like receptor 9-mediated signaling: a shared mechanism in the pathogenesis of liver inflammation and injury 47

1.3.4.1 The role of TLR9 signaling in alcoholic and non-alcoholic steatohepatitis 49

1.3.4.2 The role of TLR9 signaling in paracetamol-induced liver injury 49

1.3.4.3 TLR9-induced loss of TLR tolerance in the pathogenesis of liver transplant rejection 50

2. Specific aims **51**

3. Methods **52**

3.1 General methods **52**

3.2 Specific methods **52**

3.2.1 Methods related to genetic susceptibility to alcoholic liver cirrhosis 52

3.2.2 Methods related to the role of IRF3 in the pathogenesis of alcohol-induced liver injury 53

3.2.3 Methods related to the role of probiotics in the pathogenesis of non-alcoholic steatohepatitis 56

3.2.4 Methods related to the role of Type I interferons the pathogenesis of TLR9-associated liver injury 57

4. Results and discussion **59**

4.1 Specific aim A - The role of allelic variants in TLR-induced signaling in genetic susceptibility to alcoholic liver cirrhosis 59
4.1.1 Association of allelic variants involved in TLR4-mediated inflammatory signaling with alcoholic liver cirrhosis 59
4.1.1.1 Haplotype analysis 61
4.1.1.2 Single locus analysis 61
4.1.1.3 Validation of positive findings 62
4.1.2 Allelic variants in genes involved in the protection against oxidative stress 62
4.1.3 Association of the TNF-A -238G/A allelic variant with alcoholic liver cirrhosis (meta-analysis) 64
4.1.4 Discussion 64
4.2 Specific aim B - The cell-specific role of interferon regulatory factor (IRF3) in the pathogenesis of alcoholic liver disease 67
4.2.1 Summary of results 67
4.2.2 Discussion 68
4.2.2.1 The role of macrophage-specific IRF3 in induction of pro-inflammatory cytokines 68
4.2.2.2 Activation of IRF3 and induction of IFN-β in hepatocytes 69
4.2.2.3 The role of crosstalk between hepatocytes and immune cells in anti-inflammatory signaling in the liver 70
4.2.2.4 Incomplete protection from alcoholic liver injury in mice with bone marrow-specific deficiency in IRF3 72
4.2.3 Conclusion 74
4.3 Specific aim C - The role of probiotics in modulation of fibrosis in non-alcoholic steatohepatitis 75
4.3.1 Summary of results 75
4.3.2 Discussion 75
4.3.2 Conclusion 76
4.4 Specific aim D – The protective role of Type I interferons in TLR9-associated liver injury 77
4.4.1 Summary of results 77
4.4.3 Discussion 78
4.4.2.1 The TLR9-associated liver injury used in our research project 78
4.4.2.2 The contribution of IRF7 and IFNAR1 to the sensitization of hepatocytes to cell death 79
4.4.2.3 Cell types recruited into the TLR9-associated liver inflammatory infiltrates 80
4.4.2.4 Mechanisms of sensitization of TLR9+TLR2 ligands to LPS 81
4.4.2.5 The role of dendritic cells in liver damage/inflammation/fibrosis 81
4.4.3 Conclusion 82
5. Conclusions 83
6. References 85
7. List of publications, awards and presentations 94
8. Enclosures 103

Abbreviations

ALC	alcoholic liver cirrhosis
ALD	alcoholic liver disease
ALT	alanine aminotransferase
BPI	bactericidal/permeability increasing protein
cDC	conventional dendritic cells
CpG DNA	2'-deoxyribo(cytidine-phosphate-guanosine) DNA
DAMP	damage-associated molecular patterns
DC	dendritic cells
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Flt3	FMS-like tyrosine kinase 3
GFAP	glial fibrilar acidic protein
GFP	green fluorescence protein
HMGB-1	high mobility box 1 protein
HSC	hepatic stellate cells
HWE	Hardy-Weinberg equilibrium
IFN	interferon
IFNAR	interferon receptor
IKK-β	inhibitor of nuclear factor kappa-B kinase subunit beta
IL-1ra	interleukin 1 - receptor antagonist (protein)
IL1RN	interleukin 1 - receptor antagonist (gene)
IL-1β	interleukin 1 beta
IRAK	interleukin 1 receptor-associated kinase
IRF3	interferon regulatory factor 3
IRF3-KO	IRF3-deficient mice
IRF3-KO/WT-BM	IRF3-deficient mice with transplanted wild-type bone marrow
ISG	interferon-stimulated gene
ISGF3	IFN-stimulated gene factor 3
IκB	inhibitor of kappa B
LBP	lipopolysaccharide-binding protein
LD	linkage disequilibrium

LDH	lactate dehydrogenase
LMNCs	liver mononuclear cells
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MAPK	mitogen-activated protein kinase
MCD	methionine-choline deficient diet
MCS	methionine-choline supplemented diet
MCP-1	monocyte chemoattractant protein 1
MDA-5	melanoma differentiation-associated gene 5
MyD88	myeloid differentiation factor 88
NAPQI	N-acetyl-p-benzoquinone-imine
NASH	non-alcoholic steatohepatitis
NF-κB	nuclear factor kappa B
NK	natural killer cells
OR	odds ratio
PAMPs	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cells
polyI:C	polyinosinic-polycytidylic acid
PPAR	peroxisome proliferator-activated receptor
PRD I	IRF-binding site positive regulatory domain I
RIG-I	Retinoic acid inducible–gene I
RIP1	receptor interacting protein 1
ROS	reactive oxygen species
SOD2	superoxide dismutase 2
STAT	signal transducers and activator of transcription
TAB	TAK1-binding protein
TBK1	TANK-binding kinase 1
TGF-β	transforming growth factor beta
Th1	T helper type 1 lymphocytes
TIR	toll/interleukin 1 receptor domain
TIRAP	toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	toll-like receptor
TNF-R1	TNF-α receptor, type I

TNF-α	tumor necrosis factor alpha
TRADD	TNFR type 1-associated death domain protein
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing interferon-β
UCP2	uncoupling protein 2
WT	wild-type
WT/IRF3KO-BM	wild-type mice with transplanted bone marrow deficient in IRF3

Summary

Recent reports suggest that a majority of chronic and acute liver diseases share a significant degree of liver inflammation and injury attributable to innate immunity, activated through Toll-like receptors (TLRs). In our research, we investigated the effects of downstream signaling mechanisms triggered by TLRs on the pathogenesis of alcohol-induced liver damage, non-alcoholic steatohepatitis and immune-mediated liver injury.

In a multicentric study in Central European region involving more than 370 patients with alcoholic liver cirrhosis (ALC) and 700 controls, we investigated genetic susceptibility to ALC. We asked whether the risk of ALC could be modified by allelic variants in key molecules involved in TLR4 signaling, which is induced by the gut-derived lipopolysaccharide (LPS). We did not find any consistent association of any alleles under the study with ALC. Statistically significant association of one of the investigated alleles was found only after our data were included in a meta-analysis. However, the relative risk attributable to this allelic variant was below any biological importance. Our data indicated that the contribution of the studied alleles to genetic susceptibility to ALC is low, and suggested existence of other TLR-dependent mechanisms that could mediate the damaging effect of alcohol/LPS in the liver.

Therefore, using a mouse model, we investigated the role of the interferon-regulatory factor 3 (IRF3) in the pathogenesis of alcohol-induced liver injury. IRF3, an alternative downstream mediator of TLR4 activation, activated independently of the MyD88 adaptor, induces inflammatory cytokines and Type I interferons (IFNs). We found that IRF3 deficient mice were protected from alcohol-induced liver injury. As IRF3 is expressed both in liver macrophages and hepatocytes, we generated chimeric mice with selective *Irf3* deficiency in bone-marrow derived cells or in liver parenchymal cells. We identified that the pro-inflammatory effect of IRF3 in alcoholic liver injury is specific to bone marrow-derived cells, supporting the crucial role of TLR4/IRF3-mediated inflammation in alcohol-induced liver injury. In contrast, we showed that IRF3 in parenchymal liver cells has a protective role in alcoholic liver injury. This effect is most likely mediated by hepatocyte-derived Type I IFNs that induce Type I IFN-dependent anti-inflammatory cytokines in liver mononuclear cells which in turn modulate the extent of liver inflammation and injury.

Having demonstrated the differential effects of TLR4 signaling in the liver, we asked whether TLR4 downstream pathways could be amenable to therapeutic intervention in

NASH, which is critically dependent on gut-derived LPS. We hypothesized that modification of gut microflora by a probiotic diet would ameliorate diet-induced NASH in mice. Administration of the VSL#3 probiotic diet failed to prevent liver steatosis or inflammation. In contrast, VSL#3 ameliorated liver fibrosis in NASH. Analysis of fibrogenic pathways revealed a modulation of transforming growth factor-β signaling and collagen expression in the liver by the VSL#3 diet. These results suggested that the benefit of the VSL#3 probiotic treatment on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver.

In addition to TLR4 activation, a number of liver diseases, including autoimmune, exhibit induction of inflammatory TLR9 signaling, triggered by DNA from gut-derived bacteria or from dying host cells. In addition to inflammatory cytokines, activation of TLR9 induces a strong Type I IFN response. In a mouse model of TLR9-associated liver injury, we showed that genetic deficiency of Type I IFN induction or signaling exaggerated liver damage and inflammation, and increased production of TNF-α by liver mononuclear cells. Mice deficient in Type I IFNs showed decreased expression of the interleukin 1-receptor antagonist (IL-1ra), which is a Type I IFN-inducible anti-inflammatory cytokine. IL-1ra protected cultured hepatocytes from IL-1β-mediated sensitization to cytotoxicity from TNF-α. Moreover, administration of exogenous Type I IFN or IL-1ra ameliorated TLR9-associated liver injury, implying that the endogenous anti-inflammatory signaling induced by Type I IFNs and mediated by IL-1ra regulates the extent of TLR9-induced liver damage.

In conclusion, our data demonstrate a cell-specific role of IRF3 in the pathogenesis of alcohol-induced liver injury and support the importance of the TLR4-dependent/MyD88-independent signaling in alcoholic liver disease. Furthermore, our novel findings emphasize the active role of hepatocytes in modulating the extent of the innate immune response in the liver. Also, we demonstrate that the endogenous anti-inflammatory signaling induced by Type I IFNs regulates the extent of liver damage induced by the innate immunity, and support the indispensable role of Type I interferon signaling in TLR-mediated liver injury. Lastly, we suggest a potential role for IL-1ra in therapy of liver diseases with inflammatory component induced by TLR signaling.

Shrnutí

Společným jmenovatelem většiny chronických a akutních onemocnění jater je aktivace mechanismů vrozené imunity, které přispívají k rozvoji zánětu a poškození jaterního parenchymu. Klíčovou úlohu v rozvoji jaterního poškození hrají Toll-like receptory (TLR), jejichž signální mechanismy jsme zkoumali v souvislosti s alkoholickou a nealkoholickou nemocí jater a s imunitně zprostředkovaným jaterním poškozením.

V rámci geneticko-epidemiologické studie jsme testovali hypotézu, zda je dědičná predispozice k rozvoji ALC ovlivněna alelickými variantami v signalizačních drahách zprostředkovaných receptorem TLR4. Receptor TLR4 je aktivován bakteriálním lipopolysacharidem (LPS), který u alkoholiků ve zvýšené míře proniká z trávicího traktu do jater. Neprokázali jsme jednoznačnou asociaci žádné ze studovaných alelických variant s ALC. Zařazení našich dat o meta-analýzy sice vedlo ke zjištění statisticky signifikantní asociace jedné ze studovaných variant, avšak relativní riziko spojené s nosičstvím této alely bylo klinicky nevýznamné. Naše data ukazují, že příspěvek studovaných alelických variant k ALC je malý, avšak nevylučují, že genetická predispozice k ALC nemůže být zprostředkovaná jinými mechanismy závislými na TLR signalizaci.

Z tohoto důvodu jsme na myším modelu analyzovali význam interferonu regulujícího faktoru (IRF3) v patogenezi alkoholického poškození jater. IRF3, který je alternativním intracelulárním adaptérem receptoru TLR4, indukuje zánětlivé mediátory a interferony I. typu. Zjistili jsme, že zvířata postrádající gen *Irf3* byla kompletně chráněna před alkoholickým poškozením jater. Vzhledem k tomu, že IRF3 je exprimován jak v jaterních makrofázích, tak v hepatocytech, studovali jsme buněčně specifickou úlohu IRF3 za použití chimérních myší se selektivní deficiencí *Irf3* v jaterních buňkách původem z kostní dřeně nebo se selektivní deficiencí *Irf3* v buňkách parenchymatózních. Tento postup potvrdil naši domněnku, že prozánětlivý efekt IRF3 v alkoholickém poškození jater je zprostředkován buňkami původem z kostní dřeně. Narozdíl od prozánětlivé úlohy IRF3 v monocytech a makrofázích se zdá, že IRF3 v buňkách jaterního parenchymu má protizánětlivé vlastnosti. Ty jsou pravděpodobně zprostředkovány IRF3-dependentní produkcí interferonů I. typu, které indukují produkci protizánětlivých cytokinů, a tak modulují rozsah jaterního zánětu a poškození.

V následující studii jsme hledali odpověď na otázku možného ovlivnění TLR4 signalizace u NASH, jejíž patogeneze úzce souvisí se střevním LPS. Cílem studie bylo ověřit

hypotézu, že modifikace střevní mikroflóry probiotiky zabrání vzniku dietou indukované NASH. Probiotická dieta VSL#3 nezabránila vzniku steatózy a zánětu, avšak zpomalila progresi fibrózy. Analýza mechanismů fibrogeze odhalila modulaci signalizace TGF-beta a exprese kolagenu. Výsedky naznačují, že antifibrotické působení diety VSL#3 je nezávislé na stupni zánětu a steatózy.

Kromě aktivace receptoru TLR4 se v patogenezi jaterních chorob podílí receptor TLR9, který je aktivován DNA původem ze střevních bakterií, anebo eukaryotickou DNA z apoptotických buněk. Kromě indukce prozánětlivých cytokinů je signalizace zprostředkovaná TLR9 silným aktivátorem interferonů I. typu. Na myším modelu jaterního poškození vyvolané ligandy pro TLR9 jsme prokázali, že nepřítomnost interferonů I. typu výrazně zhoršuje stupeň a rozsah jaterního zánětu a poškození. Deficit interferonů I. typu byl spojen se sníženou expresí antagonisty receptoru pro interleukin 1 (IL-1ra), což je protizánětlivý protein závislý na interferonové signalizaci. Rekombinantní IL-1ra ochránil kultury primárních hepatocytů před cytotoxicitou vyvolanou prozánětlivými cytokiny, a po podání *in vivo* významně zmírnil jaterní zánět a poškození zprostředkované TLR9.

Naše výsledky prokazují buněčně-specifickou úlohu transkripčního faktoru IRF3 v patogenezi alkoholické nemoci jater a podtrhují klíčový význam aktivaci TLR4-dependentních mechanizmů v poškození jater alkoholem. Výsledky našeho výzkumu také naznačují aktivní roli hepatocytů v modulaci vrozené imunitní odpovědi v játrech. Naše data rovněž demonstrují, že rozsah imunitně zprostředkovaného jaterního poškození je regulován endogenní signalizací zprostředkovanou interferony I. typu, a naznačují potenciální úlohu pro použití IL-1ra v léčbě jaterního chorob se zánětlivou komponentou indukovanou TLR signalizací.

1. Introduction

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Liver diseases represent a significant cause of morbidity and mortality worldwide (1-3). In industrialized countries, up to 2% of all deaths are attributable to liver disease, which ranks liver diseases as cause of death to the 9th position. Among all gastrointestinal diseases, the liver disease is the 2nd leading cause of death after colorectal cancer (4).

A majority of chronic and acute liver diseases share a substantial degree of liver inflammation and injury mediated by the innate immune response (5). These conditions comprise prevalent liver diseases such as alcoholic liver disease (6), non-alcoholic steatohepatitis (7), viral hepatitis (8), primary biliary cirrhosis (9) and sclerosing cholangitis (10), paracetamol-induced liver injury (11), and autoimmune hepatitis (12). In addition, the innate immune response plays a pivotal role in the pathogenesis of liver fibrosis (13), ischemia-reperfusion injury (14) and liver graft rejection (15).

Innate immunity is the first line of defense against microbial invasion, and includes physical and chemical barriers, humoral factors, lymphocytic and phagocytic cells, and a group of pattern-recognition receptors that identifies specific signature molecules expressed on invading pathogens. The examples of pattern-recognition receptors include a group of Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs) to determine the presence of pathogens. Once pathogens are identified, TLRs induce multiple signaling pathways that regulate the expression of proinflammatory cytokines and chemokines to mount protective responses against invading pathogens (16).

Given the anatomical association of the liver with the intestine and its exposure to PAMPs derived from the gut, TLRs play a major role in liver physiology and pathophysiology (17). Widely expressed on liver non-parenchymal and parenchymal cells, TLRs are of a paramount importance in the initiation and progression of liver inflammation, which is an inseparable component of both acute and chronic liver injury (16).

So far, 10 human and 12 murine functional TLRs have been identified, with TLR1 – TLR9 being conserved in both species (18). Mouse TLR10 is not functional because of retrovirus insertion (19), and TLR11 and TLR12 have been lost from the human genome (20). The existence of a large number of TLRs enables the innate immune system to discriminate between PAMPs that are characteristic of different microbial classes and that include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes such as bacteria, viruses, parasites and fungi (18).

The liver is constantly exposed to small amounts of microbial components derived from the intestine. These substances are recognized by the TLR-expressing cells in the liver, but do not induce liver inflammation or injury, presumably due to low baseline expression of TLRs contributing to high tolerance of the liver to TLR ligands (21). However, disruption of the intestinal barrier, resulting in increased liver exposure by microbial components, together with external insults to the liver, such as exposure to ethanol, autoimmune insult or viral infection, compromise the liver TLR tolerance. As a result, the innate immune response is activated, resulting in liver inflammation and damage which, if sustained, proceeds into fibrosis and cirrhosis (22).

Our research has focused at two categories of TLR-induced liver diseases. The first research subject has been the alcoholic liver disease (ALD) and the non-alcoholic steatohepatitis (NASH), two major causes of liver cirrhosis which are critically dependent on signaling mediated by the TLR4. Within ALD, we asked whether variants in genes encoding crucial components of the TLR4 signaling could be instrumental in evaluation of individual susceptibility to ALD in humans (23). As for NASH, we investigated whether TLR4 signaling could be amenable to therapeutic modulation in mice. These projects have been followed by a set of experiments aimed at cell-specific role of TLR-mediated signals in the pathogenesis of ALD. These studies showed that the LPS-TLR4 signaling in hepatocytes may be protective in ALD, and suggested an important anti-inflammatory role of Type I interferons (Type I IFNs) (24). The important protective role of Type I IFNs in liver diseases was subsequently confirmed in the second range of experiments, focused on liver injury mediated by the TLR9 signaling, which is a common pathogenic mechanism of numerous liver diseases (25).

Table 1. General information on Toll-like receptors (TLRs)

	Reference
TLRs are a cornerstone of the innate immune system and provide an almost instant anti-microbial response to fight pathogens	(26, 27)
TLRs are pattern recognition receptors that detect the presence of minute amounts of signature molecules present in pathogens (pathogen-associated molecular patterns (PAMPs))	(28, 29)
Activation of TLRs activates antiviral and pro-inflammatory signaling pathways	(30, 31)
TLRs signal through the adaptor molecules myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor inducing IFN-β (TRIF) or both to activate the "MyD88-dependent" and "MyD88-independent" signaling pathways	(18, 32)
It has been suggested that TLRs may also be activated by endogenous ligands. Many of these endogenous ligands are associated with injury and inflammation and belong to a group of molecules termed damage-associated molecular patterns (DAMPs).	(33)

1.1. The role of Toll-like receptors in innate immunity

1.1.1. Structure and ligands for cell surface Toll-like receptors

Toll-like receptors are structurally characterized by the presence of a leucine-rich repeat domain in their extracellular portion and a toll/interleukin 1 receptor (TIR) domain in their intracellular domain (34). They are largely divided into two subgroups depending on their cellular localization and respective PAMP ligands. One group is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, which are expressed on cell surfaces and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins; the other group is composed of TLR3, TLR7, TLR8 and TLR9, which are expressed exclusively in intracellular vesicles such as the endoplasmic reticulum, endosomes, lysosomes and endolysosomes, where they recognize microbial nucleic acids.

TLR4, a founding member of the TLR family, was identified as the receptor that responds to bacterial lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria that can cause septic shock (35). In mice and humans, TLR4 is critical for host defense against Gram-negative bacteria (35). TLR4 forms a complex with MD2 on the cell surface, and together they serve as the main LPS-binding component (36). Binding of LPS to the TLR4/MD2 complex initiates signal transduction by recruiting intracellular adaptor molecules (Fig. 1). Additional proteins such as LPS-binding protein (LBP) and CD14 are also involved in LPS binding (36). LBP is a soluble plasma protein that binds LPS, and CD14 is a glycosylphosphatidylinositol-linked, leucine-rich repeat-containing protein that binds LBP and delivers LPS-LBP to the TLR4-MD2 complex.

TLR2 is involved in the recognition of a wide range of PAMPs derived from bacteria, fungi, parasites and viruses (35). These include lipopeptides from bacteria, peptidoglycan and lipoteichoic acid (LTA) from Gram-positive bacteria, and lipoarabinomannan from mycobacteria. TLR2 generally forms heterodimers with TLR1 or TLR6 (Fig. 1).

TLR5 recognizes the flagellin protein component of bacterial flagella (35), especially in lamina propria dendritic cells in the small intestine, which promote the differentiation of IL-17-producing helper T cells (T_H17) and T helper type 1 (T_H1) cells, as well as the differentiation of naïve B cells into immunoglobulin A-producing plasma cells in response to flagellin (37).

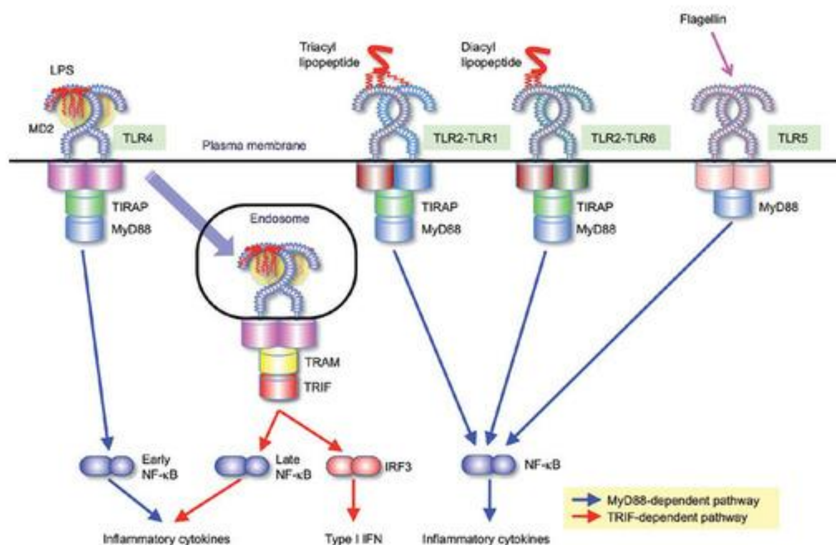


Fig. 1. PAMP recognition by cells surface Toll-like receptors. TLR4 in complex with MD2 engages LPS. Five of the six lipid chains of LPS bind MD2 and the remaining lipid chain associates with TLR4. The formation of a receptor multimer composed of two copies of the TLR4-MD2-LPS complex initially transmits signals for the early-phase activation of NF-κB by recruiting the TIR domain–containing adaptors TIRAP (Mal) and MyD88 (MyD88-dependent pathway). The TLR4-MD2-LPS complex is then internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 and late-phase NF-κB for the induction of type I interferon (TRIF-dependent pathway). Both early- and late-phase activation of NF-κB is required for the induction of inflammatory cytokines. TLR2-TLR1 and TLR2-TLR6 heterodimers recognize triacylated and diacylated lipopeptide, respectively. Two of the three lipid chains of the triacylated lipopeptide interact with TLR2, and the third chain binds the hydrophobic channel of TLR1 (absent from TLR6). TLR2-TLR1 and TLR2-TLR6 induce NF-κB activation through recruitment of TIRAP and MyD88. TLR5 recognizes flagellin and activates NF-κB through MyD88. Figure adapted from (18).

1.1.2. Structure and ligands for nucleic acid-sensing Toll-like receptors

TLR3 was originally identified as recognizing a synthetic analogue of double-stranded RNA (dsRNA), polyinosinic-polycytidylic acid (poly(I:C)), which mimics viral infection and induces antiviral immune responses by promoting the production of both Type I IFN and inflammatory cytokines. The recognition mechanism was elucidated by structural analysis of the human TLR3 ectodomain bound to dsRNA (38) (Fig. 2). In addition to recognizing poly(I:C), TLR3 recognizes the genomic RNA of reoviruses, dsRNA produced during the replication of single-stranded RNA viruses, and certain small interfering RNAs (39). TLR3 triggers antiviral immune responses through the production of the Type I IFN and inflammatory cytokines, which suggests that TLR3 has an essential role in preventing virus infection.

TLR7, originally identified as recognizing imidazoquinolone derivatives such as imiquimod and resiquimod (R-848) and guanine analogs such as loxoribine, recognizes ssRNA derived from vesicular stomatitis virus, influenza A virus and human immunodeficiency virus (40). There is a high expression of TLR7 on plasmacytoid dendritic cells (pDC) that are able to produce large amounts of Type I IFN after virus infection, and cytokine induction by pDCs in response to RNA viruses is totally dependent on TLR7 (35, 40), which suggests that TLR7 serves as the sensor of infection with ssRNA viruses.

TLR8 is physiologically most similar to TLR7. Human TLR8 mediates the recognition of R-848 and viral ssRNA. In contrast to mice that lack TLR7, mice that lack TLR8 respond normally to these agonists (41). TLR8 is expressed in various tissues, with its highest expression in monocytes, and is upregulated after bacterial infection.

TLR9 recognizes unmethylated 2'-deoxyribo(cytidine-phosphate-guanosine) (CpG) DNA motifs that are frequently present in bacteria and viruses (Fig. 2). Synthetic CpG oligonucleotides function as TLR9 ligands and directly activate dendritic cells (DCs), macrophages and B cells, and drive strong T_H1 reponses (35). There is a high expression of TLR9 by plasmacytoid dendritic cells (pDCs), and it serves as a sensor of DNA virus infection (35, 40). Recently, it has been shown that TLR9 can be activated also by DNA derived from apoptotic mammalian cells (42).

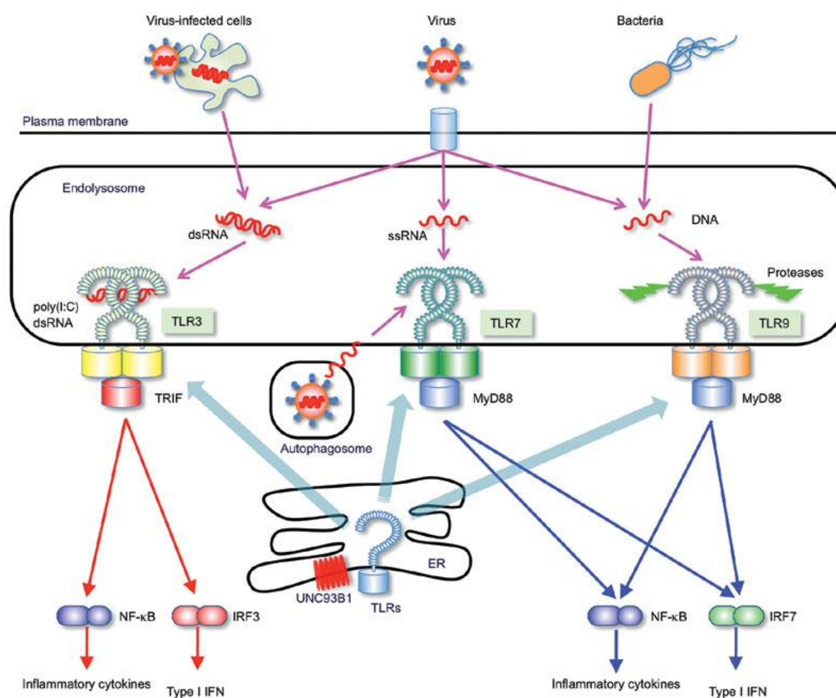


Fig. 2. PAMP recognition by intracellular Toll-like receptors. TLR3 recognizes dsRNA derived from viruses or virus-infected cells; dsRNA binds to N- and C-terminal sites on the lateral side of the convex surface of the TLR3 ectodomain, which facilitates the formation of a homodimer via the C-terminal region. TLR3 activates the TRIF-dependent pathway to induce type I interferon and inflammatory cytokines. In pDCs, TLR7 recognizes ssRNA derived from ssRNA viruses in endolysosomes and activates NF-κB and IRF7 via MyD88 to induce inflammatory cytokines and type I interferon, respectively. In addition, autophagy is involved in delivering ssRNA to TLR7-expressing vesicles. TLR9 recognizes DNA derived from both DNA viruses and bacteria. Proteolytic cleavage of TLR9 by cellular proteases is required for downstream signal transduction. TLR9 recruits MyD88 to activate NF-κB and IRF7 in pDCs. TLR3, TLR7 and TLR9 localize mainly to the ER in the steady state and traffic to the endolysosomes, where they engage with their ligands. UNC93B1 interacts with these TLRs in the ER and is essential for their trafficking. Figure adapted from (18).

1.1.2.1. Cellular localization of nucleic acid-sensing Toll-like receptors

Nucleic acid-sensing TLRs localize to various intracellular compartments. The finding that blockade of endolysosome acidification prevents TLR7- and TLR9-induced responses suggests that the delivery of internalized nucleic acids to the endolysosomes is pivotal to interaction with these TLRs. TLR9 and TLR7 are exclusively sequestered in the endoplasmic reticulum in unstimulated cells and rapidly traffic to endolysosomes after ligand stimulation (43). (Fig. 2).Mice with defective endomosomal trafficking due to a missense mutation in the gene encoding endosomal protein UNC93B1 (Fig. 2) have defects in cytokine production and upregulation of costimulatory molecules in response to TLR7 and TLR9 ligands, as well as TLR3 ligands, and are highly susceptible to viral and bacterial infection (44). In addition, macrophages deficient in gp96, a member of the endoplasmic reticulum-resident heat-shock protein 90 family, have defects in cytokine induction in response to agonists for TLR1, TLR2, TLR4, TLR5, TLR7 and TLR9.

TLR9 is proteolytically cleaved by intracellular proteases in endolysosomes, which generate a functional receptor that mediates ligand recognition and initiates signal transduction. The proteases that potentially mediate TLR9 cleavage include cathepsins and asparaginyl endopeptidase (18) (Fig. 2).

1.1.2.2 Endogenous ligands for Toll-like receptors

It is becoming increasingly evident that, in addition to responsing to PAMPs, TLRs respond to endogenous host molecules and trigger inflammatory responses. Most of these are produced as a result of cell death and tissue injury; hence their name “Damage-associated molecular patterns (DAMPs)”. They include degradation products of the extracellular matrix, heat-shock proteins and high-mobility box 1 (HMGB1) proteins, which act as stimulators for cell surface TLRs. Furthermore, chromatin-DNA and ribonucleoprotein complexes released

by dying cells and immune complex-containing self antigens, all of which contain self nucleotides, can stimulate intracellular TLR7 and TLR9 and lead to the development of systemic autoimmune disease.

As a result of injury or inflammation, components of extracellular matrix are cleaved by cellular proteases and are released outside cells. Some of the released components activate TLR2 and TLR4. These include biglycan, hyaluronic acid, versican, fibronectin and surfactant protein A (18). Biglycan induces the production of inflammatory cytokines and chemokines, and this induction is totally abolished by deficiency in both TLR2 and TLR4. Hyaluronic acid fragments can stimulate macrophages to produce chmokines through TLR2 and TLR4.

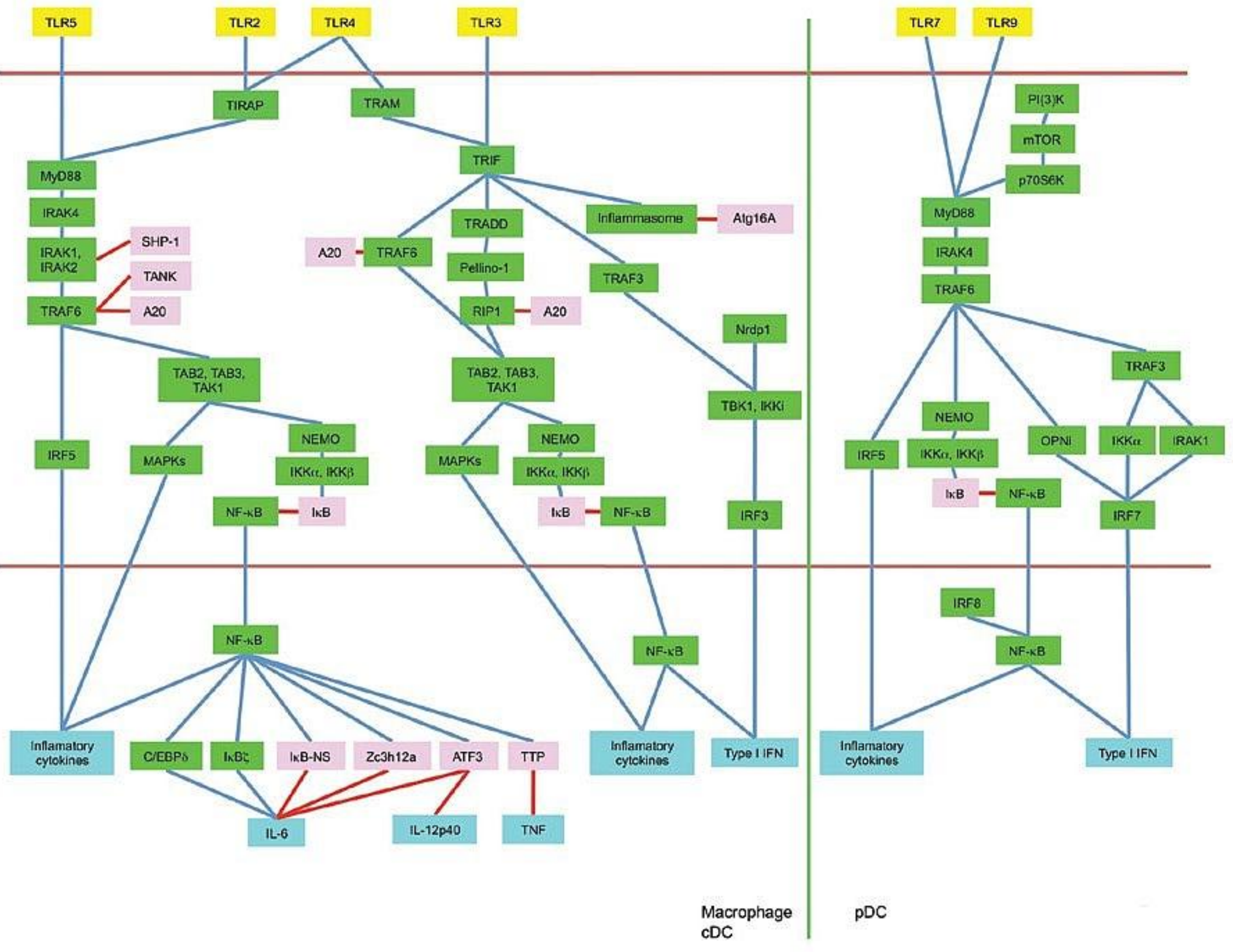
In addition to ECM components, other cellular components such as HMGB1 and heat-shock proteins serve as ligands for TLR2, TLR4 and TLR9 (45). HMGB1, a nuclear non-histone protein that is released by necrotic cells or during inflammation, is a proinflammatory mediator in septic shock and in ischemia-reperfusion models. Neutralizing antibodies to HMGB1 inhibit damage in a hepatic ischemic reperfusion model, and *Tlr4*-deficient mice show less damage in this model (46). This finding suggests that TLR4 reponds to endogenous molecules and mediates inflammatory responses in a noninfectious situation.

1.1.3. Major intracellular pathways involved in Toll-like receptor signaling

Individual TLRs trigger specific biological responses. For example, TLR3 and TLR4 generate both Type I IFNs and inflammatory cytokines, whereas cell surface receptors TLR1-TLR2, TLR2-TLR6 and TLR5 induce mainly inflammatory cytokines. These differences are explained by the discovery of TIR domain-containing adaptor molecules, including MyD88, TIRAP (Mal), TRIF and TRAM, which are recruited by distinct TLRs and activate distinct signaling pathways (Fig. 3). MyD88, the first identified member of this TIR family, is used by all TLRs except TLR3, and activates the transcription factor nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines (35). In contrast, TRIF is used by TLR3 and TLR4 and induces alternative pathways that lead to activation of the transcription factors IRF3 and NF-κB and to consequent induction of type I interferon and inflammatory cytokines. Thus, TLR signaling pathways can be largely classified as either MyD88-dependent pathways, which drive the induction of inflammatory

cytokines, or TRIF-dependent pathways, which are responsible for the induction of type I interferon as well as inflammatory cytokines (47).

TLR4 is the only TLR that activates both the MyD88-dependent and TRIF-dependent pathway (Fig. 3). TLR4 initially recruits TIRAP at the plasma membrane and subsequently facilitates the recruitment of MyD88 to trigger the initial activation of NF-κB and MAPK (48). TLR4 subsequently undergoes dynamin-dependent endocytosis and is trafficked to the endosome, where it forms a signaling complex with TRAM and TRIF, rather than TIRAP and MyD88, to initiate the TRIF-dependent pathway that leads to IRF3 activation as well as the late-phase activation of NF-κB and MAPK (49). Thus, TLR4 activates the MyD88-dependent pathway earlier than the TRIF-dependent pathway. Notably, activation of both the MyD88- and TRIF-dependent pathways is necessary for the induction of inflammatory cytokines via TLR4 signaling, which is in contrast to other TLRs, for which activation of either the MyD88- or the TRIF-dependent pathway is sufficient for the induction of inflammatory cytokines.



TLR5
TLR2
TLR4
TLR3
TLR7
TLR9
TIRAP
TRAM
TRIF
PI(3)K
mTOR
p70S6K
MyD88
IRAK4
IRAK1, IRAK2
SHP-1
TANK
TRAF6
A20
TRADD
Pellino-1
RIP1
Inflammasome
Atg16A
TRAF3
Nrdp1
TBK1, IKKi
TAB2, TAB3, TAK1
IRF5
MAPKs
NEMO
IKKα, IKKβ
NF-κB
IκB
IRF3
OPN
IKKα
IRAK1
IRF7
IRF8
Inflamatory cytokines
C/EBPδ
IκBζ
IκB-NS
Zc3h12a
ATF3
TTP
Type I IFN
IL-6
IL-12p40
TNF
Macrophage
cDC
pDC

Figure 3. Overview of TLR signaling pathways. TLR-mediated responses are controlled mainly by the MyD88-dependent pathway, which is used by all TLRs except TLR3, and the TRIF-dependent pathway, which is used by TLR3 and TLR4. TRAM and TIRAP are sorting adaptors used by TLR4 and TLR2-TLR4, respectively. In conventional dendritic cells (cDC) and macrophages, MyD88 recruits IRAK4, IRAK1, IRAK2 and TRAF6 and induces inflammatory responses by activating NF-κB, MAPK and IRF5. TRAF6 activates TAK1 in complex with TAB2 and TAB3 and activates the IKK complex consisting of NEMO and IKKα/β, which catalyze phosphorylation of IκB proteins. NF-κB induces C/EBPδ, IκBζ, IκB-NS, Zc3h12a, ATF3 and tristeraprolin (TTP), which influence the genes encoding IL-6, IL-12p40 or TNF-α. TRIF recruits TRAF6, TRADD and TRAF3. TRADD interacts with Pellino-1 and RIP1. RIP1 and TRAF6 cooperatively activate TAK1, which leads to activation of MAPK and NF-κB. TRAF3 activates the kinases TBK1 and IKKi, which phosphorylate and activate IRF3, the last of which controls transcription of type I interferon. Nrdp1 is involved in TBK1-IKKi activation. The TRIF-dependent pathway leads to inflammasome activation during TLR4 signaling. In pDCs, TLR7 and TLR9 recruit MyD88 along with IRAK4 and TRAF6, which activate IRF5 and NF-κB for inflammatory cytokine induction and IRF7 for type I interferon induction. For IRF7 activation, IRAK1- and IKKα-dependent phosphorylation is required, and TRAF3 is located upstream of these kinases. The PI(3)K-mTOR-p70S6K axis enhances the TLR7 and TLR9 signaling pathways. IRF1 is involved in the induction of type I interferon by TLR7 and TLR9 in cDCs rather than plasmacytoid dendritic cells (pDCs). Among the many negative regulators of TLRs that have been identified, TANK (which suppresses TRAF6), A20 (which suppresses TRAF6 and RIP1), ATG16A (which suppresses inflammasome activation) and SHP-1 (which suppresses IRAK1 and IRAK2) are reported to be indispensable for preventing inflammatory diseases caused by enhanced or prolonged TLR signaling. Yellow, TLRs; green, stimulators; pink, negative regulators; blue, target genes. Figure adapted from (18)

1.1.3.1. The MyD88-dependent pathway in Toll-like receptor-induced signaling

After the engagement of TLRs by their cognate PAMPs, MyD88 recruits the IL-1 receptor-associated kinases IRAK4, IRAK1, IRAK2 and IRAK-M (Fig. 3). IRAK4 is activated initially and has an essential role in the activation of NF-κB and MAP downstream of MyD88 (35). IRAK1 and IRAK2 are activated sequentially, and activation of both kinases is required for robust activation of NF-κB and MAPK (50). IRAK activation results in an interaction with TRAF6, an E3 ligase that catalyzes the synthesis of polyubiquitin linked to Lys63 (K63) on target proteins, including TRAF6 itself and IRAK1. The K63-linked polyubiquitin chains then bind to the zinc finger-type ubiquitin-binding domain of TAB2 and TAB3, the regulatory components of the kinase TAK1 complex, to activate TAK1. The K63-linked polyubiquitin chains also bind to the ubiquitin-binding domain of NEMO, and essential regulatory component of the IKK complex required for NF-κB activation. Thus, the K63 polyubiquitin chains might be responsible for recruiting TAK1 to form a complex with IKK, thus allowing TAK1 to phosphorylate the inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) through its close proximity to the IKK complex, which leads to phosphorylation and subsequent degradation of inhibitors of kappa B (IkB) proteins, and thus enables activation of the NF-κB (51). Activation of the MyD88-dependent pathway also results in genes that have critical roles in modulating NF-κB-dependent transcription. These genes encode the IkB protein IkBξ, C/EBPδ, IkB-NS and ATF-3 (Fig. 3).

1.1.3.2. The MyD88-independent/TRIF-dependent pathway in Toll-like receptor-induced signaling

The TRIF-dependent pathway culminates in the activation of both IRF3 and NF-κB (39) (Fig. 3). TRIF recruits TRAF6 and activates TAK1 for NF-κB activation, most probably through ubiquitination-dependent mechanisms similar to those of the MyD88-dependent pathway. TRIF also recruits the adaptor RIP1 through the distinct RIP homotypic interaction motif. RIP1 undergoes K63-linked polyubiquitination after stimulation by TLR3 agonists, and this modification is required for NF-κB activation. The adaptor TRADD binds RIP1, and TRADD-deficient cells show impaired RIP1 ubiquitination with concomitant loss of NF-κB activation (52), which suggests involvement of TRADD in RIP1 activation downstream of TLR3. Collectively, TRIF forms a multiprotein signaling complex along with TRAF6, TRADD, Pellino-1 and RIP1 for the activation of TAK1, which in turn activates the NF-κB and MAPK pathways.

In addition to leading to NF-κB activation, the TRIF-dependent pathway leads to IRF3 activation and interferon-β transcription (Fig. 3).TRIF recruits a signaling complex involving the noncanonical IKKs TBK1 and IKKi (IKK-ε), which catalyze the phosphorylation of IRF3 and induce its nuclear translocation (53). The activation of TBK1-IKKε by TRIF requires TRAF3. TRAF3 deficiency impairs interferon-β induction by various nucleic acid-sensing receptors (54).

1.1.4. Activation of the Interferon regulatory factors and Type I Interferons via the Toll-like receptor signaling

The key factors in IFN induction are the interferon regulatory factors (IRF). The IRFs are transcription mediators of virus-, bacteria- and IFN-induced signaling pathways and play a critical role in antiviral defense, immune responses, cell growth regulation and apoptosis. To date, nine human cellular IRF genes have been indentified (IRF-1, IRF-2, IRF3, IRF-4/Pip/ICSAT, IRF-5, IRF-6, IRF7, ICSBP/IRF-8 and ISGF3/p48/IRF-9) (55). These factors all share significant homology in the N-terminal 115 amino acids, which contains the DNA-binding domain. All IRFs with the exception of IRF-1 and IRF-2 contain the IRF associated domain which mediates these interactions in the 3’-terminal part of the protein. The

availability of genetically modified mice, which have distinct IRF deleted, has revealed that the function of IRF is not limited to the induction of Type I IFN genes (Table 2).

Table 2. Phenotypic changes in IRF null mice. IRF3 and IRF7, whose roles have been studied within our research projects, are highlighted in bold.

IRF	Defects	Reference
IRF-1	Apoptosis, iNOS, IL-12	(56)
IRF-2	NK cell deficiency and inhibition of NK cell maturation; development of mDCs	(57)
IRF3	**Down-modulation of Type I IFN induction; increased susceptibility to infection**	(58, 59)
IRF-4	T, B cell maturation	(60)
IRF-5	Induction of inflammatory cytokines TNF-α, IL-6 and IL-12	(61)
IRF-6	Embryonic lethal, differentiation of keratinocytes	(62)
IRF7	**Block in Type I IFN induction**	(59, 63)
IRF-8	Differentiation of pDC, induction of IL-12, IL-23	(64)
IRF-9	Type I and II IFN signaling, induction of IRF7, IFN-α and ISG	(65)

1.1.4.1. Interferon regulatory factor 3 (IRF3) and 7 (IRF7)

The identification of IRF3 and IRF7 in transcriptional activation of Type I IFN genes had a major impact of the understanding of the molecular mechanism of the pathogen induced innate antiviral responses (66). It became obvious that although pathogen recognition may be mediated by distinct cellular receptors and signaling pathways, they all lead to the activation of IRF3 and IRF7, which are critical for the transcriptional activation of Type I IFN genes (35) (Fig. 4).

The ubiquitously expressed **IRF3** (67) is activated in infected cells upon recognition of dsRNA, which has been considered the common signature of virus-infected cells. TLR3 or the cytoplasmic RNA helicases RIG-I and MDA-5 are important for the recognition of most RNA virus infections (35, 68). The TLR3, RIG-I/MDA-5 signaling pathways, and TRIF-dependent TLR4-mediated pathway lead to the phosphorylation of IRF3 at the C' terminal region, where serine 386 is critical for activation by the two non-canonic IκB kinases, TBK-1

and IKKε (69). Crystal structure analysis shows that phosphorylation results in the structural changes which allow IRF3 activation (70). The activated IRF3 then homodimerizes with another IRF3 or heterodimerizes with IRF7 and translocates to the nucleus, where it stimulates transcription of *IFN-B* as well as some interferon stimulated genes (ISG), such as *RANTES*, *ISG15* and *ISG56* (71). While expression of IRF3 alone is sufficient to activate the promoter of the *IFN-B* gene (72), the *IFN-B* enhanceosome contains not only IRF3 but also IRF7 recognition elements (58, 73).

IRF7 was initially identified as a factor binding to the promoter of the Epstein-Barr virus and a splice variant of IRF7 was recognized as a factor that plays a critical role in the induction of *IFN-A* genes (66). Reconstitution of IRF7 expression in infected human fibroblasts which expressed only IFN-β conferred expression of several *IFN-A* genes (74). Mice with homozygous deletion of *IRF7* were unable to express Type I IFN genes upon viral infection or activation of TLR9 by CpG-rich DNA, indicating that IRF7 is a master regulator of Type I IFN expression (63). Like IRF3, IRF7 is phosphorylated by the TLR3, TLR-7 and TLR9 mediated signaling pathways in which serines 477 and 479 appear to be critical targets for activation by TBK-1 (75). In contrast, TLR7- and TLR9-stimulated phosphorylation of IRF7 is dependent not on TRIF/TBK-1 but rather on MyD88 and IκB (76) and involves formation of ternary complex containing MyD88, IRAK-4, IRAK-1 and TRAF6 (77). Virus-induced expression of distinct IFN-A subtypes is determined by the organization of the IRF3 and IRF7 recognition domains in the virus responsive element of the *IFN-A* promoters. The differential expression of the individual IFN-A subtypes has been shown to be due to a distinct nucleotide substitution in the domains (78) and by the presence of negative regulatory sequences located in the upstream regulatory region of some IFN-A subtypes (79). IRF3 and IRF7, together with histone transacetylases, have been shown to be part of the transcriptionally active human *IFNA1* enhanceosome (80), whereas the murine *Ifna11* promoter, which is not activated by IRF3, binds only IRF7 homodimers (78). These data indicate that the relative levels of IRF3 and IRF7 in cells determine the levels of expression of individual IFN-A subtypes. IRF7 was shown to have a short half-life which may play a role in the regulating the transient expression of *IFN-A* genes (58).

1.1.4.2. TLR7 and TLR9 signaling in plasmacytoid dendritic cells

The TLR7 and TLR9 signaling pathways in plasmacytoid dendritic cells (pDCs) have been extensively investigated to elucidate their potential to induce the production of type I

interferon after viral infection. The TLR7 and TLR9 signaling pathways in pDCs are unique in that they both require MyD88 for the induction of Type I IFN (Fig. 4). In this context, IRF7, which is constitutively expressed by pDCs, binds MyD88 and forms a multiprotein signaling complex with IRAK4, TRAF6, TRAF3, IRAK1 and IKKα (39) (Fig. 4). In this complex, IRF7 becomes phosphorylated by IRAK1 and IKKα, dissociates from the complex and translocates into the nucleus. In addition to requiring phosphorylation, IRF7 activation requires TRAF6- and Ubc13-dependent ubiquitination. In addition, the phosphoinositol 3-OH kinase (PI(3)K) and mTOR are required for nuclear translocation of IRF7 and induction of Type I IFN (81).

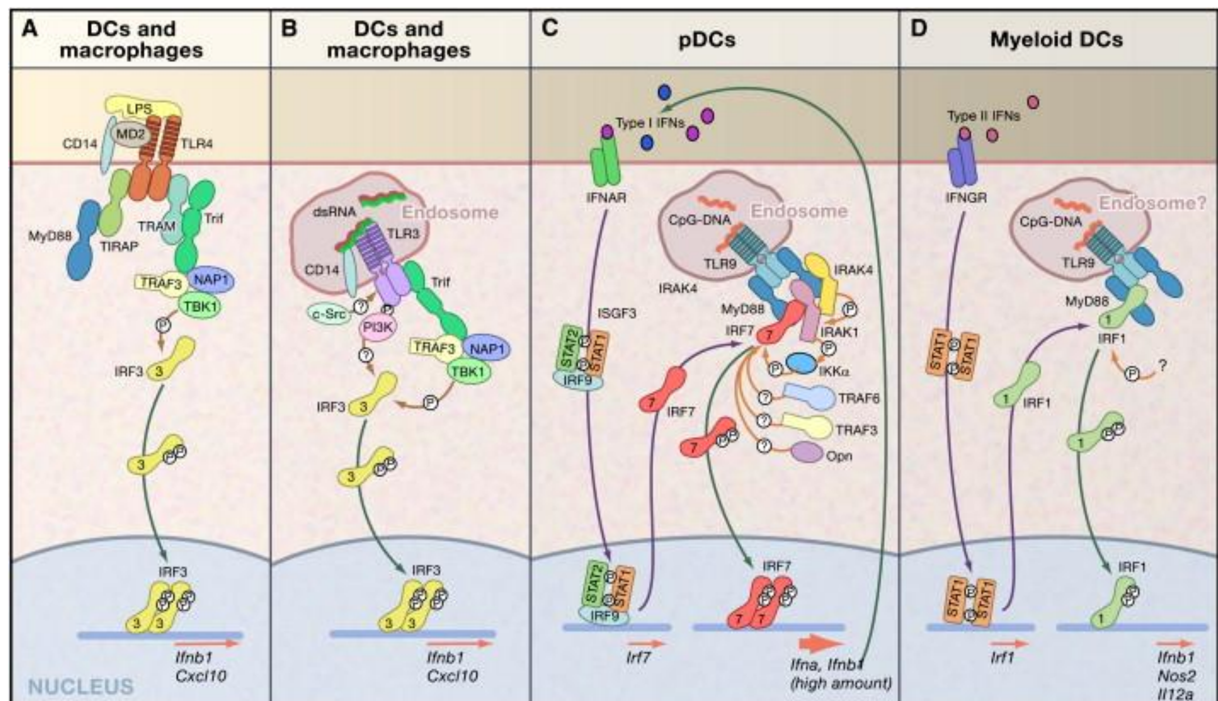


Figure 4. TLR-Mediated Type I IFN Induction Pathways. (A) The receptor complex composed of TLR4, MD-2, and CD14 recognizes LPS and signals through at least four adaptors: TIRAP, MyD88, TRAM, and Trif. Among them, TRAM and Trif mediate the activation of IRF3. Trif associates with TBK1 through TRAF3 and NAP1, which mediates the phosphorylation of IRF3. Phosphorylated IRF3 forms homodimers and induces IFN-β and Cxcl10 genes. **(B)** TLR3 (expressed in endosomes) activates IRF3 through a pathway similar to that utilized by TLR4. PI3K is recruited to the phosphorylated tyrosine residues of TLR3 and supports the activation of IRF3. The tyrosine kinase c-Src also associates with TLR3 and is involved in the activation of IRF3; however, the precise role of c-Src remains to be clarified. **(C)** Upon TLR7 or TLR9 (expressed in endosomes) stimulation, IRF7 interacting with MyD88 is activated by the IRAK4-IRAK1-IKKα kinase cascade. The exact function of the other molecules depicted here is not known. Secreted type I IFNs enhance the expression of IRF7 gene, leading to further enhancement of type I IFN gene induction. **(D)** IFN-γ stimulation induces the expression of IRF1 via the formation of homodimers of STAT1. Induced IRF1 interacts with and is activated by MyD88 by an as yet unknown mechanism and translocates to the nucleus to induce IFN-β, iNOS, and IL-12p35 genes. All pathways depicted here operate in a cell type-specific manner. Figure adapted from (68).

Retention of the CpG-TLR9 signaling complex in the endosome is also an important mechanism used by pDCs to control antiviral innate immune responses. The A (D)-type CpG oligodeoxynucleotide, which contains a single CpG motif and a poly(G) tail on phosphorothioate-phosphodiester backbone, is able to induce the secretion of Type I IFN by pDCs. It is stably retained in the early endosomes in pDCs along with TLR9, MyD88 and IRF7 (63). In contrast, the B (K)-type CpG oligonucleotide, which contains multiple CpG motifs on a phosphothiotate backbone can induce both IL-12 production by cDCs and B cell activation, is rapidly transferred to late endosomes or lysosomes, which results in less activation of IRF7.

1.1.4.3. The family of Interferons

Interferons, so called because of their activity to interfere with virus replication in the cell, are divided into at least three distinct types: types I, II and III (82). Type I IFNs are composed of various genes including IFN-α and IFN-β (83), and others, such as IFN-ω, IFN-ε and IFN-κ (82). In humans and mice, the IFN-α genes are composed of more than 13 subfamily genes (13 in humans and 14 in mice), whereas only a single IFN-β gene is found (84).

Type II IFN is referred as IFN-γ, the gene which exists in a single copy; this gene is structurally unrelated to Type I IFNs and is typically induced in cells of the immune system such as T cells or NK cells (85). Recently, some new IFN gene members, namely IFN-λ1, -λ2 and –λ3, also known as IL-29, IL-28A and IL-28B, respectively, have been indentified and classified as type III IFN (82).

1.1.4.3.1. Plasmacytoid dendritic cells as the major producers of Type I IFNs

Although virtually all cells can produce Type I IFNs in response to viral or bacterial pathogens, pDCs are the most potent, producing up to 1000-fold more Type I IFNs than other cell types (86). Functionally, immature pDCs exhibit low allostimulatory activity and can even be tolerogenic (87). Activation of immature pDCs with viruses, CpG DNA, IL-3 or CD-40 ligand allows them to become mature, expressing high levels of class I and class II MHC, costimulator CD80 and CD86 molecules, as well as CD8α, thereby resembling conventional DCs (88). The number of pDCs in lymphoid organs is very low, and there is considerable mouse strain variation (89). A substantial recruitment of these cells in bone marrow and spleen can be achieved by the Flt3-ligand (90).

The most distinguishing feature of human and mouse pDCs is their production of large amounts of Type I IFNs in the precursor stage. This production is further enhanced upon stimulation of endosomal TLRs (TLR7 and TLR9) with ssRNA, CpG DNA, or certain types of autoantigen-autoantibody immune complexes. The reason for the extraordinary production of Type I IFNs by pDCs may be explained by a high constitutive expression of IRF7, compared to other cell types (91).

1.1.4.3.2. Induction of Type I IFNs: a two-step hypothesis

It has been shown that IRF3 is expressed constitutively in a variety of cells and localizes in the cytoplasm as an inactive monomer (92). Upon phosphorylation at the phosphorylation sites in the C-terminal region (Ser385, 386, 396, 398, 402 and 405), activation of IRF3 and homodimerization ensues (93). The dimeric form of IRF3 then translocates to the nucleus, forms a complex with the p300/CBP co-activator and binds to the PRD I or PRD III element (18) (Fig. 5).

IRF3 was thought to be primarily responsible for the initiation of IFN-β induction: the IFN-β gene is first activate by signals that induce the cooperative binding of IRF3 with other transcription factors, namely NF-κB, c-Jun/ATF2, to the *IFNB* promoter, resulting in initial induction of relatively small amounts of IFN-β. This initial induction of IFN-β triggers a positive amplificatory loop by binding to Type I IFN receptors and a strong induction of Type I IFNs (second phase), mediated by IRF7, which can activate both IFN-α and IFN-β genes (94) (Fig. 5). Recent findings suggest that in addition to IRF3 homodimers, the IRF7 homodimers and IRF3/IRF7 heterodimers are also critical for activating the initial phase of IFN-β induction (55). Once the initial activation of IFN genes is achieved by IRF3 and IRF7, the positive feedback becomes fully operational, wherein the IFN-induced IRF7 fully participates.

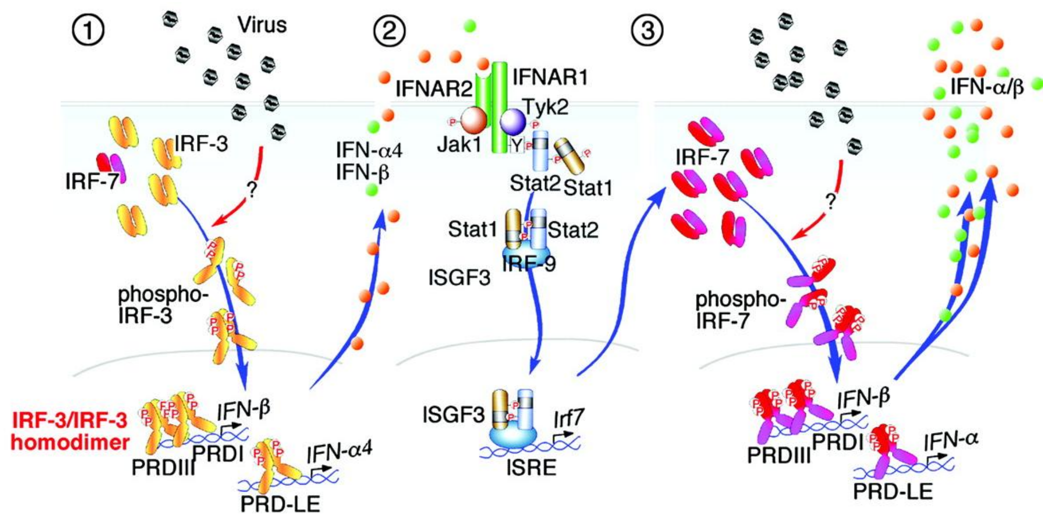


Fig. 5. Positive amplificatory loop in Type I interferon induction. The initial induction of IFN-β is initiated through cytoplasmatic RNA receptors, such as RIG-I, or through membrane-bound receptors, such as TLR3 or TLR4. Signals from these receptors to the *IFNB* promoter is mediated by IRF3/IRF3 homodimers and by IRF3/IRF7 heterodimers. Secreted IFN-β binds to Type I IFN receptors (IFNAR1/IFNAR2) and in autocrine or paracrine way activates the transcription complex ISGF3, composed of Stat1/Stat2/IRF-9. The ISGF3 transcription complex induces IRF7, which in turn strongly activates genes encoding IFN-β and IFN-α. Adapted from (55).

1.1.4.3.3. Signaling triggered by Type I Interferons

IFN-α and IFN-β share a ubiquitously expressed heterodimeric receptor IFNAR composed of IFNAR1 and IFNAR2 subunits. Evidence suggests that IFNAR2 serves as the ligand-binding chain, but both chains are required for signal transduction (95). In general, various cell types display small numbers of these high-affinity receptors. IFN-α or IFN-β binding leads to ligand-induced receptor dimerization, followed by phosphorylation of the two receptor-associated Janus protein tyrosine kinases (Tyk2 on IFNAR1 and Jak1 on IFNAR2) (96). Phosphorylation of the Jak kinases results in phosphorylation of the intracellular domain of IFNAR1 and creation of docking sites for STAT2 (preassociated with STAT1 on IFNAR2), which is phosphorylated and serves as a platform for recruitment and phosphorylation of STAT1 (97). The phosphorylated STAT1/STAT2 heterodimers then dissociated from the receptor and translocate into the nucleus through an unknown mechanism, where they associated with the IFN regulatory factor 9 (IRF-9, also referred to as p48) to form the heterotrimeric complex IFN-stimulated gene factor 3 (ISGF3) (98). The ISGF3 binds to upstream regulatory consensus sequences (IFN-stimulated response elements – ISRE) on Type I IFN-inducible genes and initiates transcription (99).

1.2. Expression of Toll-like receptors in the liver

Due to its anatomical links to the gut, the liver is constantly exposed to gut-derived bacterial products, and functions as a major filter organ and a first line of defence. Eighty percent of intravenously injected endotoxin is detected in the liver within 30 minutes (100). Moreover, the liver is an important site for bacterial phagocytosis and clearance as it hosts more than 80% of body macrophages. Kupffer cells, the resident macrophages of the liver, are able to efficiently take up endotoxin and phagocytose bacteria carried through the portal vein and are considered to play a major role in the clearance of systemic bacterial infection (101). The healthy liver contains low mRNA levels of TLRs such as TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, TLR10 and signaling molecules such as MD-2 and MyD88 in comparison to other organs (102), suggesting that the low expression of TLR signaling molecules may contribute to the high tolerance of the liver to TLR ligands from the intestinal microbiota to which the liver is constantly exposed.

Kupffer cells, the resident macrophages of the liver, play a crucial role in host defence which is linked to their ability to phagocytose, process and present antigen, and secrete various pro-inflammatory mediators including cytokines, prostanoids, nitric oxide and reactive oxygen species (103). Kupffer cells are among the first cells in the liver to be hit by gut-derived toxins such as LPS and orchestrate inflammatory responses within the liver. Accordingly, Kupffer cells express TLR4 and are responsive to LPS (104). Although some studies have demonstrated that Kupffer cells are involved in the uptake and hepatic excretion of LPS (105, 106), others have shown that Kupffer cell depletion does not reduce LPS clearance, most likely due to the ability of hepatocytes to uptake and to eliminate LPS into the bile (107). Moreover, Kupffer cells can inactivate LPS by deacetylation (108). Following stimulation with LPS at concentrations between 0.1 – 1000 ng/mL, Kupffer cells produce TNF-α, IL-1β, IL-6, IL-12, IL-18, IL-10 and several chemokines (109). Kupffer cell-derived IL-12 and IL-18 activate hepatic natural killer (NK) cells to increase the synthesis and release of antimicrobial IFN-γ (110). Notably, Kupffer cells mediate the majority of cytokine and chemokine expression in liver after LPS injection, as demonstrated by depletion experiments (111). Moreover, Kupffer cells stimulated profibrogenic response by the production of transforming growth factor beta 1 (TGF-β1), matrix metalloproteinases, platelet-derived growth factor, and reactive oxygen species (112). Kupffer cells also functionally express TLR2, TLR3 and TLR9 (110). In comparison to peripheral blood monocytes, Kupffer cells

express low levels of CD14 (113). Moreover, freshly isolated human Kupffer cells secrete the anti-inflammatory cytokines IL-10 in response to stimulation with LPS, which contributes to the downregulation of pro-inflammatory cytokines (114). Thus, Kupffer cells may have a higher LPS tolerance to adapt to the special circumstances in their anatomical location that is frequently hit with low levels of LPS even under normal conditions.

Hepatocytes express TLR4 receptors and are responsive to LPS, but this response is fairly weak with only 2-fold induction of most upregulated genes in a microarray after stimulation with LPS. Moreover, dose of 100 ng/mL and higher were required to see significant induction of the NF-κB-dependent and MAPK-dependent signaling in hepatocytes (115). Similarly, stimulation with TLR2 ligands induces NF-κB activation (116). The expression of TLR2 in hepatocytes is upregulated by LPS, TNF-α, bacterial lipoprotein and IL-1β in and NF-κB dependent manner indicating that hepatocytes become more responsive to TLR2 ligands under inflammatory conditions (116). In contrast, TLR4 expression in hepatocytes is not upregulated by proinflammatory mediators (117).

Hepatocytes play a major role in the uptake of LPS and its removal from the systemic circulation by secreting LPS into the bile (105, 107). Clearance of LPS occurred at a similar rate in rats that had been depleted of Kupffer cells by gadolinium chloride indicating that hepatocytes are the principal mediators of this process (107). A recent study demonstrated that TLR4, CD14 and MD-2 are required for the uptake of LPS by hepatocytes (118). Interestingly, TLR4 signaling is not required for this process as hepatocytes from *Tlr4*-deficient C3H/HeJ mice were as efficient as those isolated from TLR4-sufficient C3H/HeOuJ mice to take up LPS (118).

Hepatic stellate cells. Following liver injury, hepatic stellate cells (HSCs) undergo an activation process and become the predominant extracellular matrix-producing cell type in the liver (119). Activated human HSCs express TLR4 and CD14 and respond to LPS with the activation of NF-κB and JNK as well as the secretion of pro-inflammatory cytokines (120). Activated mouse HSCs express TLR2, TLR4 and TLR9 and respond to LPS, lipoteichoic acid and CpG-DNA with an upregulation of extracellular-related kinase (Erk) phosphorylation and IL-6, TGF-β1 and monocyte chemoattractant protein-1 (13, 121, 122). Quiescent murine HSCs express as much TLR4 as *in vivo*-activated HSCs and are highly responsive to LPS, even at low doses (1 ng/mL) (13). Moreover, quiescent HSCs activate NF-κB in response to LPS injection *in vivo*. Notably, LPS downregulates the TGF-β pseudoreceptor Bambi in quiescent HSCs to promote TGF-β signaling and stellate cell activation (13).

Biliary epithelial cells. Mouse biliary epithelial cells express CD14, MD-2 and TLR2, TLR3, TLR4 and TLR5 (123), and display NF-κB activation and TNF after high-dose LPS stimulation (123). Human biliary epithelial cells express TLR1-10 (124).

Sinusoidal endothelial cells form the fenestrated lining of the hepatic sinusoids and thus have an important function in hepatic perfusion and nutrient supply. Sinusoidal endothelial cells constitutively express TLR4 and CD14 as well as TLR9, and show an increase in NF-κB activation after LPS stimulation (125, 126). Moreover, mRNA for TLR1-9 was detected in sinusoidal endothelial cells, and functional expression of TLR3, but not of TLR4, has been demonstrated by the ability of supernatants from poly(I:C)- treated sinusoidal cells to reduce hepatitis B virus replication in immortalized hepatocytes (127). After repetitive LPS challenges, sinusoidal endothelial cells showed reduced NF-κB activation, CD54 expression and a reduced ability to promote leukocyte adhesion (125). In sinusoidal endothelial cells, LPS tolerance is not regulated at the level of TLR4 surface expression, but appears to be linked to prostanoid expression (125). The role of sinusoidal endothelial cells in the hepatic uptake of LPS is currently unclear (105, 107).

Hepatic dendritic cells (DCs) are the professional antigen-presenting cells of the liver. Plasmacytoid DCs (pDCs, CD11c+B220+), but not conventional DCs (cDCs, CD11c+B220-), are the principal cells that produce IFN-α in response to the ligands for TLR9 and TLR7, but not TLR4 (89). Hepatic pDCs produce TNF-α and IL-6 in response to the ligands for TLR2, TLR3 and TLR4 (128). Both pDCs and cDC subsets upregulate co-stimulatory molecules (CD40, CD80 and CD86) in response to TLR4, TLR7 and TLR9 (89). Of note, hepatic DCs are hyperresponsive to TLR ligands to produce TNF-a, IL-6 and IL-12, but less capable of inducing TLR4-mediated and TLR9-mediated allogenic T cell proliferation compared with splenic DCs (128). Thus, hepatic DCs have unique properties that enable to induce strong innate responses with a lower capability of allostimulation.

Other types of immune cells in the liver. Liver natural killer (NK) cells synthesize high amounts of IFN-γ in response to IL-12 synergistically with IL-18 (129). Liver NK express TLR1, TLR2, TLR3, TLR4, TLR6, TLR7 and TLR9 and respond to corresponding TLR agonists synergistically with IL-12 to produce IFN-γ and chemokines, such as CCL3, CCL4 and CCL5 (130). In general, T cells are indirectly activated by TLRs through APC-mediated IL-12 and IFN-α, which induce Th1 polarization (131). There is limited evidence that T cells directly respond to LPS to enhance their adhesion (132).

1.3. Toll-like receptors in the pathogenesis of liver diseases

During the past few decades, a rapid progress has been made in understanding the role of innate immune system liver physiology and pathophysiology. This advancement has been made possible by the key findings that: 1. identified the liver as a crucial organ involved in response to microbial components (100, 133); 2. demonstrated that numerous cell types in the liver express TLRs and respond to TLR ligands (summarized in (22, 112)) ; 3. reported increased exposure of the liver by pathogen- and damage-associated molecular patterns in many types of chronic liver diseases (summarized in (16)), and 4. documented that absence of TLR ligands or deficiency in their recognition prevented numerous types of liver diseases in animal models (134-139). (Table 3).

Table 3: The current concept for the role of TLRs in liver diseases

	Reference
The liver is a target of bacterial TLR ligands due to its anatomical connection with the intestine	(100, 101, 133)
Under normal circumstances, the liver is exposed to small amounts of bacterial PAMPs, but does not show signs of inflammation due to its higher tolerance to PAMPs and its ability to efficiently excrete PAMPs such as LPS	(105, 107)
In many types of liver diseases, levels of PAMPs are elevated. Most research has focused on LPS, and has shown increased LPS levels in chronic viral hepatitis, liver fibrosis and cirrhosis, and alcoholic liver disease	(16)
LPS promotes liver injury and fibrogenesis under many circumstances. Blocking LPS release from the intestinal microbiota, or inhibiting activation and signaling of the LPS receptor TLR4 may therefore represent a feasible strategy for the prevention or treatment of chronic liver disease.	(134-136, 140)

Toll-like receptors proved to be essential in pathogenesis of alcoholic liver disease (141), non-alcoholic steatohepatitis (7), hepatitis B and C (142, 143), primary biliary cirrhosis (9) and sclerosing cholangitis (10), paracetamol-induced liver injury (11), and autoimmune hepatitis (12). In addition, innate immunity plays a pivotal role in the pathogenesis of liver fibrosis (13, 144, 145), ischemia-reperfusion injury (14) and liver graft rejection (15).

In the following sections, we will provide a detailed account on liver injury mediated by TLR4 signaling, such as ALD and NASH, and on immune-mediated liver injury induced by TLR9 signaling, which have been the focus of our research. Information on the role of TLRs in other liver diseases that were not included in our research projects can be found elsewhere (16, 22, 112, 141).

1.3.1. Toll-like receptors in the pathogenesis of alcohol-induced liver injury

Alcohol abuse is a leading cause of morbidity and mortality worldwide (146), and ALD, ranging from steatosis, steatohepatitis to fibrosis and cirrhosis, accounts for up to 50% of deaths from cirrhosis in Western world (147).

The pathogenesis of ALD involves both liver parenchymal and non-parenchymal cells, including resident and recruited immune cells that contribute to liver damage and inflammation (148). The concept of dysregulated innate immunity as an indispensable component of alcohol-induced liver disease dates back to the observations that patients with ALD have increased antibodies against *Escherichia coli* in plasma (149), and that chronic alcohol administration increases gut-derived endotoxin in the portal circulation, activating resident liver macrophages to produce several proinflammatory cytokines (150). Recognition of Toll-like receptors (TLR) as the key components involved in activation of the innate immune system enabled a substantial progress in understanding of the mechanisms mediating alcohol-induced liver injury. The current concepts for the pathogenesis of alcohol-induced liver injury are summarized in Figure 5.

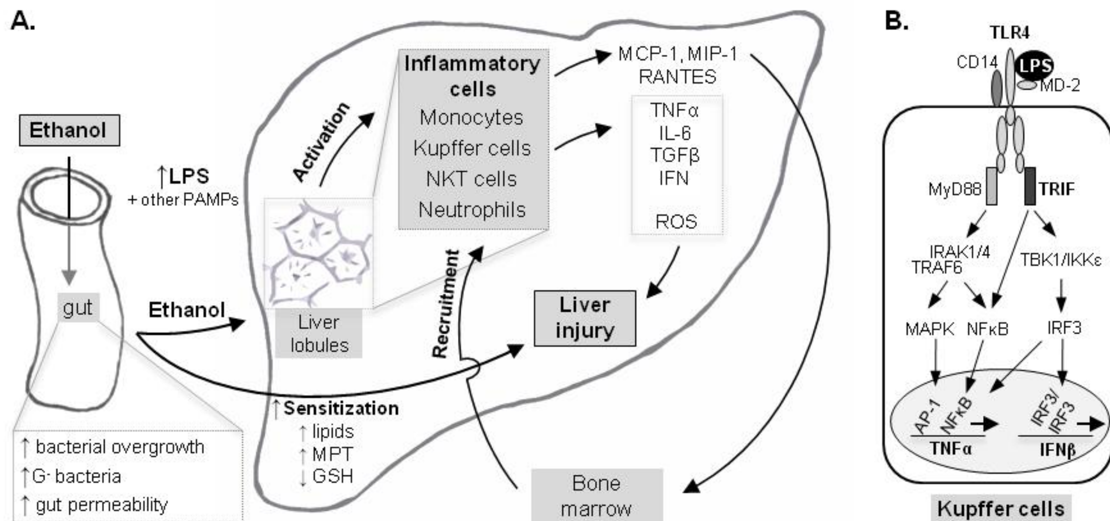


Fig. 5. Pathophysiology of TLR-mediated alcohol-induced liver injury.
(A) Ethanol promotes translocation of LPS and other pathogen-associated molecular patterns (PAMPs) from the gut to the portal vein and to the liver. In the liver, LPS induces activation and recruitment of bone marrow-derived inflammatory cells. Activated bone marrow-derived cells synthesize inflammatory cytokines and reactive oxygen species that induce liver injury. Chronic ethanol per se contributes to sensitization of monocytes/macrophages to LPS, and to sensitization of hepatocytes to the cytotoxic effect of inflammatory cytokines. The latter is brought about by accumulation of lipids, opening of mitochondrial permeability transition (MPT) pores and depletion of glutathione (GSH). **(B)** In macrophages/Kupffer cells, TLR4 recognizes LPS in cooperation with its co-receptors, CD14 and MD-2. The signal is passed through MyD88-dependent or TRIF-dependent intracellular pathways, which activate various transcription factors, including AP-1, NF-κB and IRF3, and induces pro-inflammatory cytokine and Type I interferon genes.

1.3.1.1. Gut-derived bacterial components are critical in the pathogenesis of ALD

Due to its unique anatomy and blood supply the liver receives blood from the intestine, exposing hepatocytes and cells in the liver sinusoids not only to nutrients but also to gut-derived microbial products. The gut mucosal epithelium serves as an interface between the vast microbiota and internal host tissues (151). Under normal circumstances, a normal balance of gut barrier function, gut permeability and equilibrium of commensal and pathogenic microorganisms in the gut lumen is maintained and mostly prevents microbial translocation from the gut (152). LPS (endotoxin), a component of Gram negative bacterial wall and other components derived from bacteria in the intestinal microflora normally penetrate the mucosa only in trace amounts, enter the portal circulation and are cleared by 80-90% in the liver through uptake by Kupffer cells (resident liver macrophages) and hepatocytes in a manner that prevents cell damage or inflammation (153). This physiological uptake and detoxification is important for preventing systemic reactions to gut-derived bacterial components.

Multiple lines of evidence support the hypothesis that gut-derived endotoxin is involved in alcoholic liver injury. First, it has been shown that excessive intake of alcohol

increases gut permeability of normally nonabsorbable substances (154). Second, intestinal Gram-negative bacteria, as well as blood endotoxin, are increased in acute (155) and chronic (156) alcohol feeding models. Patients with alcoholic fatty liver, alcoholic hepatitis and alcoholic cirrhosis have 5- to 20-fold increased plasma endotoxin compared to normal subjects (157). Third, intestinal sterilization with antibiotics (136) and displacement of Gram-negative bacteria with *Lactobacillus* treatment (158) prevented alcohol-induced liver injury. The mechanism underlying the disruption of the intestinal barrier appears to be multifactorial (159). Disruption of tight junctions has been attributed to acetaldehyde (152) and liver-derived inflammatory cytokines, particularly TNF-α, that enter the systemic circulation and further disrupt tight junctions, thus perpetuating intestinal barrier dysfunction (160). Gut permeability may be also increased by ethanol-induction of miR212, a microRNA that downregulates proteins of the zona occludens in intestinal cell culture and that was increased in colonic biopsy samples in patients with ALD (161).

Activation of Kupffer cells has been identified as one of the key elements in the pathogenesis of alcohol-induced liver damage. Kupffer cells are the largest population of tissue macrophages, predominantly distributed in the lumen of hepatic sinusoids and exhibit endocytic activity against blood-borne materials entering the liver (162). Triggering of toll-like receptor signaling drives Kupffer cells to produce inflammatory cytokines and chemokines and to initiate the inflammatory cascade (22). Indeed, the essential role of Kupffer cells as a central component of the pathomechanism of ALD has been demonstrated in studies in mice and rats that show that inactivation of Kupffer cells with gadolinium chloride or liposomal clodronate can almost fully ameliorate alcohol-induced liver disease (135, 137).

1.3.1.2. Recognition of Toll-like receptor ligands in alcohol-induced liver injury

Activation of Kupffer cells via TLR4-dependent mechanism plays a crucial role in the pathogenesis of alcohol-induced liver injury (136, 150, 163). LPS, a component of Gram-negative bacteria, is a potent activator of innate immune responses through its binding to the TLR4 complex, and comprises three distinct parts: a carbohydrate (O-antigen), the oligosaccharide core region and a lipid portion (Lipid A). Only the lipid A portion is immunogenic (164). While TLR4 cannot directly bind LPS, the co-receptors CD14 and MD-2 bind LPS and upon LPS binding activate TLR4. CD14 is a GPI-anchored protein, which also exists in soluble form, and facilitates the transfer of LPS to the TLR4/MD-2 receptor

complex that modulates LPS recognition (165). MD-2 is a soluble protein that non-covalently associates with TLR4 and binds LPS directly to form a complex with LPS in the absence of TLRs (166). The association between LPS and CD14 is facilitated by LPS-binding protein (LBP), which is a soluble shuttle protein (167). TLR4, CD14 and LBP are critical in alcohol-induced liver injury. Alcoholic liver injury was prevented in C3H/HeJ mice (134), which have functional mutation in the TLR4 gene and have defective response to bacterial endotoxin (168). Prevention of alcohol-induced liver inflammation and injury in C3H/HeJ mice was associated with decreased TNF-α expression, compared to wild-type mice. Similar protection from alcohol-induced liver injury was observed in mice deficient for *Lbp* (139) and *CD14* (169), whereas mice transgenic for human CD14 were hypersensitive to LPS (170).

Since disruption of intestinal barrier by ethanol increases permeability for macromolecular substances in general (152), it is likely that other bacterial components, in addition to LPS, are translocated to the portal blood in alcoholics. In particular, bacterial DNA was found in serum and ascites of patients with advanced liver cirrhosis leading to increased cytokine production in peritoneal macrophages (171, 172). Bacterial DNA, which is detected by TLR9, sensitizes the liver to injury induced by LPS via upregulation of TLR4, MD-2 and induction Th1-type immune response in the liver (173). Hepatic expression of TLR9 was increased in wild-type animals using the Lieber-DeCarli chronic alcohol feeding model, and alcohol feeding sensitized to TLR9 ligand CpG to enhance TNF-α production (174). In patients with alcoholic cirrhosis, purified B cells stimulated with TLR9 ligand CpG *ex vivo* showed significant upregulation of immunoglobulin A, compared to B cells from control individuals (175), suggesting involvement of TLR pathways in alcohol-induced hyperimmunoglobulinemia (175). Also, overexpression of TLR9, TLR4 and TLR2 was associated with impaired neutrophil function in alcoholic liver cirrhosis (176).

Taken together, it seems likely that sensitization to TLR ligands in alcohol-induced liver damage is regulated by multiple mechanisms, including those that are directly dependent on gut-derived bacterial components and TLR signaling, but also other mechanisms, such as lipid accumulation in hepatocytes (177), histone acetylation in ethanol-exposed macrophages (178) or activation of Kupffer cells by C3 and C5 components of the complement pathway (179).

1.3.1.3. Downstream effects of TLR4 signaling in alcohol-induced liver injury

Recent evidence suggests that TLR4 downstream signaling in ALD is mediated predominantly through MyD88-independent pathway, rather than through the MyD88-dependent mechanism. Alcohol feeding with the Lieber-DeCarli diet (180, 181) resulted in significant steatosis and liver damage in *MyD88*-deficient mice compared to mice on pair-fed diet and the extent of alcohol-induced changes was comparable in alcohol-fed *MyD88*-deficient and wild-type mice (182). The involvement of the MyD88-independent TLR4 signaling pathway was indicated by upregulation of IRF7, an IRF3-inducible gene, in Kupffer cells (183). In a different study it was reported that mice deficient in *Trif*, which is a key TLR4 downstream adaptor in the MyD88-independent pathway, were protected against alcohol-induced liver disease and it is likely that IRF3, a transcription factor downstream to TLR4/TRIF, binds to the TNF-α promoter resulting in induction of TNF-α (184). These findings demonstrate that TLR4-mediated signaling via MyD88-independent pathways is critical in induction of alcoholic liver disease.

1.3.1.4. Transcription factors in alcohol-induced liver injury

The importance of molecular mechanisms culminating in nuclear events leading to activation of a wide array of transcription factors in various liver cell types is widely studied in progression of alcoholic liver injury. These transcription factors bind to the promoter regions in target genes resulting in induction of cytokines, chemokines and various other mediators including kinases, adaptor proteins and receptors. Similar to alterations in transcription factors related to fatty acid metabolism, chronic alcohol induced inflammatory mediators are also modulated by key transcription factors. The most studied transcription factor is NF-κB and alteration in its DNA binding activity has been observed in livers following chronic alcohol consumption (173) as well as in isolated monocytes/macrophages (185). Chronic alcohol increased NF-κB activity in monocytes and macrophages leading to an up-regulation in various inflammatory cytokine and chemokine genes (186, 187). Another transcription factor modulated by chronic alcohol exposure is AP-1, wherein increased expression and activity was observed in livers of chronic alcohol fed mice (188). Activation of peroxisome proliferator-activated receptor gamma (PPARγ), another transcription factor, was beneficial and prevented chronic alcohol-induced liver injury in mice (189). While PPARγ is thought to be involved in anti-inflammatory cytokine production, its exact

mechanism in alcoholic livers is not known. Further, Egr-1 another zinc finger transcription factor, is up-regulated in LPS-stimulated isolated Kupffer cells from chronic alcohol-fed mice and is dependent on ERK activation (190). Egr-1 knock-out mice were protective to alcoholic liver injury, indicating a role for the Egr-1-ERK pathway in the pathogenesis of alcoholic liver injury (191).

1.3.1.5. Pro-inflammatory cytokine induction in alcoholic liver disease

Alcoholic steatohepatitis is characterized by infiltration of various inflammatory cells in the liver, including monocytes, macrophages, neutrophils and lymphocytes, which occurs as a consequence of activation of inflammatory mediators induced by TLR signaling (192, 193). In humans with alcoholic steatohepatitis, serum TNF-α, IL-6 and IL-8 levels are increased and their levels correlate with markers of the acute-phase response, liver function and clinical outcome (194). There is also evidence for activation of circulating monocytes in individuals with ALD, based on increased TNF-α production and increased NF-κB activation (195).

Induction of TNF-α by TLR4 signaling and by reactive oxygen species in Kupffer cells has been identified as a major component in ALD (196, 197). The effect of TNF-α in hepatic inflammation and hepatocyte apoptosis is mediated through TNF receptor TNF-R1 (196). Binding of TNF-α to TNF-R1 activates several signal transduction pathways (198), resulting in the activation transcription factors including NF-κB and c-Jun-N-terminal kinase (199), and in activation of pro-apoptotic Fas-associated death domain (200).

Circulating levels of TNF-α and TNF-R1 are higher in patients with alcoholic steatohepatitis than in heavy drinkers with inactive cirrhosis, heavy drinkers who do not have liver disease, and individuals with neither alcoholism nor liver disease (201, 202). High serum levels of TNF-α and TNF-R1 correlated with mortality in patients with acute alcoholic hepatitis (202, 203). Hepatic expression of TNF-R1 is enhanced in chronic ethanol consumption (204), and liver injury is substantially reduced when alcohol diet is administered in TNF receptor 1 (TNF-R1) – knockout mice or in rats that have been pretreated with anti-TNF-α antibodies or thalidomide, which reduces production of TNF-α (138, 205).

Under normal circumstances, hepatocytes are resistant to the pro-apoptotic effect of TNF-α; however, several conditions prime hepatocytes to TNF-α-mediated cell death in the setting of chronic alcohol consumption (206). Hepatocytes from rats chronically fed alcohol

have increased TNF-α induced cytotoxicity associated with mitochondrial permeability transition pore opening (206) and with a profound effect of alcohol on mitochondrial functional integrity (207). Also, decreased mitochondrial glutathione in alcohol-fed rats (208), or inhibition of hepatic transmethylation reactions by S-adenosylhomocysteine (209) has been shown to sensitize hepatocytes to TNF-α mediated cytotoxicity. Moreover, animal models of alcohol-induced liver injury show impaired function of proteasomes that increases hepatocyte sensitivity to TNF-α-mediated apoptosis (210). Interestingly, although upregulation of TNF-R1 is observed in the livers of patients with alcoholic steatohepatitis (204), a recent *in vitro* study showed that free fatty acids sensitized HepG2 cells to TRAIL-mediated apoptosis, but not to cytotoxicity mediated by TNF-α (177).

In addition to the metabolic changes involved in sensitization to TNF-α cytotoxicity, the net effect of TNF-α on hepatocytes is influenced by other cytokines. For example, deficiency of IL-10, an anti-inflammatory cytokine, exacerbates TNF-α mediated liver injury in mice by alcohol (211). Conversely, mice that are deficient in interleukin-12 (212), interferon-γ (213), or interleukin-18 (214), are protected against TNF-α -induced liver damage. The subtle balance between hepatocyte proliferation and apoptosis is also regulated by an autocrine cascade involving the pro-proliferative TGF-α and IL-1 receptor antagonist, and the anti-proliferative IL-1β (215).

1.3.1.6. Toll-like receptors and induction of oxidative stress in alcohol-mediated liver injury

Cellular responses induced by oxidative stress play an important role in innate immune cell activation. Kupffer cells produce reactive oxygen species (ROS) in response to chronic alcohol exposure as well as endotoxin (216). Interaction of NADPH with TLR4 is involved in LPS-mediated ROS generation and NF-κB activation and production of inflammatory cytokines in neutrophils (217) and in human monocytes (218). Pretreatment of chronic alcohol fed rats with inhibitor of NADPH oxidase diphenyleneiodonium normalized ROS production, decreased LPS-induced ERK1/2 phosphorylation and inhibited increased TNF-α production in Kupffer cells (216). Inhibition of NADPH oxidase prevented steatosis, upregulation of TLR2, 4, 6 and 9 mRNA, and sensitization to respective ligand-induced liver injury (174), indicating a cross-talk between oxidative stress and TLR pathways in ALD. Protection from alcohol-induced liver injury was observed in p47 phox-/- mice, deficient in

the main cytosolic component of NADPH oxidase, further supporting the important role of NADPH oxidase in alcohol-induced inflammatory response and liver injury (216).

Protection against hepatocyte damage induced by alcohol is mediated by the mitochondrial superoxide dismutase 2 (SOD2), which inactivates superoxide radicals that are increasingly generated in early phases of alcohol-induced liver injury (219). Overexpression of *SOD2* induced by *in vivo* transfection of hepatocytes using adenoviral vector prevented alcohol-induced liver injury in rats (220). The increase in mitochondria-derived reactive oxygen species (ROS) is controlled also by the uncoupling protein 2 (UCP2) (221). The *UCP2* gene is constitutively expressed in Kupffer cells, whereas no baseline expression has been described in healthy hepatocytes. Treatment with LPS decreases *UCP2* expression in Kupffer cells. In contrast, stimulation with TNF-α *in vitro* increases *UCP2* expression in hepatocytes (222). It seems likely that decreased UCP2 expression in Kupffer cells contributes to ROS production, whereas hepatocyte-specific expression of UCP2 (223) and SOD2 (224) protect hepatocytes from oxidative stress.

1.3.1.7. Cell-specific effects of the Interferon regulatory factor 3 (IRF3) in the pathogenesis of alcoholic liver disease: questions relevant to our research

The current concept of TLR4-dependent pathogenesis of ALD supports the predominant role of the MyD88-independent (TRIF-dependent) pathway (182), mediated by the transcription factors IRF3 and NF-κB. However, IRF3 has reportedly been expressed in multiple liver cells types, including Kupffer cells (non-parenchymal cells) and hepatocytes (parenchymal cells). Moreover, although IRF3 in monocytes is crucially involved in the TLR4-mediated induction of inflammatory cytokines (184), multiple reports have demonstrated that IRF3 is also involved in Type I IFN induction in hepatocytes (225-227) and. These findings suggest that both inflammatory cytokines and Type I IFNs may play a role in the pathogenesis of ALD and point at a possible cell-specific role of IRF3 in the liver. We hypothesized that IRF3 is critical in alcohol-induced liver injury. Given the differential input of parenchymal and non-parenchymal cells in pathophysiology of ALD, we further hypothesized that IRF3 may be critical in alcoholic liver injury in a cell-specific manner. Therefore, we employed a mouse model in which the effect of chronic alcohol feeding on liver damage was evaluated in animals with global deficiency of IRF3 or in animals with selective deficiency of IRF3 in liver parenchymal or non-parenchymal cells.

1.3.2. The role of genetic factors in the pathogenesis of alcoholic liver disease

1.3.2.1. Genetic susceptibility to alcoholic liver disease

Susceptibility to alcoholic liver disease has a genetic predisposition that is separate from the susceptibility to alcoholism (228). Concordance rates for alcoholic psychosis (21.1% vs. 6.0%) and liver cirrhosis (14.6% vs. 5.4%) are higher among monozygotic twins than dizygotic twins (229). Concordance for alcoholism, however, is similar among twin pairs, and the greater concordance for a pathological outcome among monozygotic twins favors a genetic predisposition for the organ-specific complications of alcoholism. The low frequency of cirrhosis among heavy drinkers of alcohol (10-20%) also suggests a separate genetic basis for a pathological outcome in some patients (230). The predilection does not refute a dose-response relationship between the amount of alcohol consumed and the risk of advanced liver disease (231), but it does suggest that host-related factors modify individual susceptibility to alcohol-related injury.

1.3.2.2. Candidate pathways for genetic susceptibility to alcoholic liver disease

Genes that modulate alcohol metabolism, fibrogenesis, display of histocompatibility antigens and lipid peroxidation, and genes encoding cytokines have been implicated in the pathogenesis of alcoholic liver disease (232). However, data that would support the role of fibrogenic genes, HLA haplotypes or genes regulating lipid peroxidation do not provide a firm evidence for their involvement in susceptibility to alcoholic liver disease (228). In addition, a recent meta-analysis showed that none of the once promising allelic variants in alcohol-metabolizing enzymes are associated with alcohol-induced liver injury (233). Taken together, it cannot be excluded that the genetic variants involved in the above mentioned mechanisms modulate alcohol-induced liver injury, but they do not seem to play an essential role in the genetic susceptibility to alcoholic liver disease.

1.3.2.3. Genetic variability in genes modulating the TLR4-induced alcoholic liver injury

The lack of a firm evidence for genetic predilection for ALD that would be conferred by genes in the alcohol metabolism, histocompatibility antigens and lipid peroxidation

suggests involvement of other pathways and genes. To exert a substantial role in genetic predisposition to ALD, these pathways would have to be of critical importance in the pathogenesis of ALD and would have to abound with gene variations that would influence expression of the respective gene or the function of the respective protein.

Looking from this perspective, the TLR4 signaling seems to be a suitable candidate, given its critical role in the pathogenesis of ALD and the fact that majority of genes coding for receptors and signaling molecules involved shows a high frequency of functionally relevant allelic variants. For example, the -159C/T variation in the promoter of *CD14* enhances its expression by monocytes (234), whereas two linked variations, c.896A/G and c.1196C/T, in the coding region of *TLR4* impede the activation of monocytes by LPS (235). In addition, the recognition of LPS by TLR4 is modulated by the *LBP* c.1306C/T and the *BPI* c.645A/G allelic variants (236). The variations -238G/A and -863C/A in the *TNFA* promoter independently increase transcription of TNF-α (237, 238). The *IL-1* gene cluster contains *IL1B* and *IL1RN* genes, which encode IL-1β and its receptor antagonist IL-1ra, respectively. The variant –31T in the promoter of *IL1B*, which is in linkage disequilibrium with the variant *IL1B* -511C, significantly increases IL-1 transcription (239). The IL1RN gene contains a penta-allelic 86-bp tandem repeat. The second most common IL1RN*2 allele containing two repeats increases the secretion rate of IL-1β *in vitro* (240).

Functional genetic variants have been described also in genes for regulators of mitochondrial oxidative stress, which is inducible by the TLR4 mechanism (chapter 1.3.1.6) and is critically involved in the pathogenesis of ALD. For example, three functional allelic variants have described in the *UCP2* gene: the promoter variant *UCP2* -866G/A, the variant 45 ins/del in 3'-untranslated region (UTR) and the *UCP2* c.164C/A in exon 4. The former two variants modulate *UCP2* expression, the latter decreases UCP2 protein activity (241-244). Only one functional allelic variant *SOD2* c.47C/T (Ala16/Val) has been reported in the mitochondrial targeting sequence of the SOD2 protein (245). The amino acid exchange alters the protein conformation and increases the efficacy of mitochondrial targeting of SOD2 (246).

1.3.3. The role of probiotics in TLR4-mediated pathogenesis of non-alcoholic steatohepatitis

Nonalcoholic fatty liver disease and its advanced stage NASH are becoming the most common causes of chronic liver disease in Western countries. Recent reports demonstrate that high fat diet and obesity alters the composition of caecal microbiota in favor of LPS-containing strains, and increases the gut permeability, resulting in increased bacterial translocation (22). The key role of LPS and TLR4 signaling in NASH is supported by reduced hepatic triglyceride content and inflammation in mice undergoing intestinal decontamination with antibiotics, or in mice deficient for *Tlr4* (16). These findings emphasize the crucial role of gut-derived bacterial products in the pathogenesis of NASH (see also chapter 1.3.4.1) and suggest that modification of intestinal microbiota by probiotics may represent a feasible approach for the prevention and treatment of NASH.

To test the hypothesis that modification of intestinal microbiota would have impact on NASH, we employed a mouse model of NASH which were administered with the VSL#3, a probiotic preparation of live, freeze-dried bacteria containing eight bacterial species (*Streptococcus salivarius* subsp. *thermophilus*, *Bifidobacterium* [*B. breve*, *B. infantis*, *B. longum*], *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus*). We asked whether the VSL#3 probiotic preparation, which has been previously shown to prevent hepatic damage and maintain colonic barrier function in a mouse model of sepsis (247), would ameliorate diet-induced NASH by modulation of liver inflammation and fibrosis.

1.3.4. The Toll-like receptor 9-mediated signaling: a shared mechanism in the pathogenesis of liver inflammation and injury

The increasing amount of information on the regulation of innate immunity in the liver suggests that individual liver diseases are unlikely to be attributable to a single signature TLR ligand; instead, multiple TLR ligands and receptors, often with overlapping signaling pathways, contribute to prevalent liver inflammation and injury (Table 4).

Table 4. The overlapping spectrum of TLRs involved in the pathogenesis of liver diseases. Involvement of pathways mediated by TLR9 is highlighted in bold.

Liver diseases	Abnormalities in expression and function of TLRs	Mechanisms of action and consequences	Reference
Alcoholic hepatitis	TLR 2, 3, 4, 6	TLR 4 – pathogenesis through gut-derived LPS	(134, 182)
	TLR 7, **9**	TLR 2, 3, 4, 5, 6, 9, CD14 – upregulation in the liver	(174, 182)
	CD14	TLR 2, 3, 4, 5, 6, 9, CD14 – increased sensitization to ligands and pro-inflammatory cytokine production	(174)
Non-alcoholic fatty liver	TLR 4, **9**	TLR 4 – pro-inflammatory cytokine production	(248, 249)
Hepatitis B	TLR 1, 2, 3, 4, 6	TLR 1, 2, 4, 6, 7, 9 – downregulation of mRNA and cytokine production	(250)
	TLR7, **9**	TLR 3, 9 – decreased HBV replication in Kupffer cells and liver sinusoidal endothelial cells	(251)
Hepatitis C	TLR 1, 2, 3, 4, 6	TLR 3 – recognition of virus, hijacked by NS3/4A	(252)
	TLR 7, 8, **9**	TLR 2 – dendritic cells, monocytes recognize NS3 and HCV core	(253)
		TLR 4 – hyperresponsive monocytes to LPS; loss of TLR tolerance	(142)
		TLR 7, 9 – decreased IFN-α by plasmacytoid dendritic cells	(253)
		TLR 2, 3, 6, 7, 8, 9 – upregulation of mRNA in monocytes and T cells	(142, 254)

Liver fibrosis	TLR 2, 4, **<u>9</u>**	TLR 2, 4, 9 – activation of stellate cells	(22)
Primary biliary cirrhosis	TLR 2, 3, 4, 5 TLR **<u>9</u>**	TLR 3 – upregulation of mRNA TLR 2, 3, 4, 5, 9 – sensitization to ligands in monocytes	(255, 256)
Primary sclerosing cholangitis	TLR 4, **<u>9</u>**	TLR 4, 9 – induction of inflammation	(10)
Acute liver failure	TLR 4, **<u>9</u>**	TLR 4, 9 – sensitization to LPS injury TLR 9 - induction of inflammation	(257) (11)
Ischemia-reperfusion injury	TLR 2, 4, **<u>9</u>**	TLR 2, 4 – activation of inflammatory responses leading to injury	(258)
Liver graft rejection	TLR **<u>9</u>**	TLR 9 – abrogation of spontaneous tolerance	(15)

It is noteworthy that all liver conditions mentioned in Table 4 show involvement of TLR9 signaling. This may seem surprising given the fact that TLR9 is an endosomal receptor activated by prokaryotic CpG-rich DNA (63). However, since disruption of intestinal barrier, observed in numerous liver diseases (22), increases permeability for macromolecular substances in general (152), it is not unexpected that other bacterial components, in addition to LPS, are translocated to the portal blood and to the liver (259). Second, it has been reported that the CpG-rich repeats that activate TLR9 are present in DNA from apoptotic mammalian cells (42), and that intraportal administration of DNA isolated from apoptotic hepatocytes induced liver injury (11). These findings suggest that TLR9-mediated signaling in the liver can be activated by gut-derived bacterial CpG-DNA and/or by DNA released from hepatocytes undergoing apoptosis as a result of a previous insult. Therefore, it seems that TLR9 may be a common denominator in different pathogenic processes that lead to liver damage.

Specifically, emerging data provide an evidence for the role of CpG DNA and TLR9-mediated inflammation in acute and chronic liver injury of diverse origin, including non-alcoholic steatohepatitis (249), acetaminophen-induced liver injury (11), alcoholic liver disease (174), primary biliary cirrhosis (9) and primary sclerosing cholangitis (10). TLR9-initiated signals are also involved in liver fibrosis (140), ischemia-reperfusion injury (14), and liver graft rejection (15). TLR9 acts synergistically with the TLR2 ligand LTA (260), and sensitizes to liver injury induced by the TLR4 ligand LPS (257).

1.3.4.1. The role of TLR9 signaling in alcoholic and non-alcoholic steatohepatitis

It is widely accepted that LPS, a gut bacteria-derive endotoxin, is important for the development and progression of ALD and NASH through TLR4 activation and induction of Kupffer cell activity (16, 22, 141). Experimental and clinical data have demonstrated that levels of circulating and hepatic LPS are elevated both in ALD and NASH. Increased LPS levels in ALD are likely owing to increased gut permeability caused by excessive alcohol intake (152), whereas in individuals with NASH, it may be related to small intestinal bacterial overgrowth and alterations of the intestinal barrier (261). In addition to TLR4, increased expression TLR9 was observed in animal models of ALD and NASH (7, 174). In addition, feeding with alcohol resulted in sensitization to liver inflammation and damage because administration of TLR4 and TLR9 ligands increased expression of TNF-α (174).

Direct evidence supports the crucial role of TLR9 signaling in the pathogenesis of NASH. Miura et al. (249) have identified TLR9 as another important player in the pathogenesis of NASH. They based their findings on a murine model of NASH induced by a choline-deficient amino acid-defined (CDAA) diet. As demonstrated in their study (249), consumption of CDAA diet activates TLR9 signaling on Kupffer cells, thereby inducing IL-1β production via a MyD88-dependent pathway, inflammation and liver injury. Additionally, IL-1β then increases lipid accumulation in hepatocytes by up-regulating diacylglycerol acyltransferase 2 and subsequently induces hepatocyte death. Produced by Kupffer cells, IL-1β was also previously shown to play an important role in the development of hepatic steatosis via down-regulating PPAR-α in a murine model using a high-fat diet (262).

1.3.4.2. The role of TLR9 signaling in paracetamol-induced liver injury

The analgesic paracetamol (acetaminophen) is widely known for its potential to cause severe and sometimes lethal liver injury. When ingested in large amounts, acetaminophen overwhelms the normal metabolic pathways of glucuronidation and sulfation and undergoes oxidation to form the highly reactive intermediate N-acetyl-p-benzoquinone-imine (NAPQI). NAPQI is not harmful if it combines rapidly with glutathione; however, when hepatic glutathione stores are depleted, NAPQI escapes detoxification, resulting in liver cell death (263). In the liver, the initial wave of drug-induced hepatocellular destruction is followed by a robust innate immune response, in which invading inflammatory cells cause a second wave

of destruction (264). This is evident from studies in a mouse strain with a mutation in *Tlr4*, in which liver disease is significantly attenuated following an acetaminophen challenge (265).

Recently, Imaeda et al. (11) demonstrated, in a mouse model, that DNA from dying hepatocytes is the trigger of the innate immune response induced by the TLR9, and that the exaggerated immune response seen in liver injury induced by acetaminophen is mediated predominantly through the inflammatory cytokines IL-1β and IL-18 in a TLR9-dependent manner. Mammalian DNA interacts specifically with TLR9, which, like all nucleic acid–sensing TLRs, is sequestered intracellularly within endosomes. TLR9 was once considered incapable of binding mammalian DNA because of its affinity for unmethylated CpG motifs characteristic of microbial DNA. DNA from injured mammalian cells, however, has the capacity to activate TLR9 (266), and recently even normal mammalian DNA has been shown to engage this receptor and stimulate an immune response (267).

1.3.4.3. TLR9-induced loss of TLR tolerance in the pathogenesis of liver transplant rejection

Rejection of liver allograft is less severe in liver transplantation than in the transplantation of other solid organs (268). In addition, human liver allografts require lower doses of immunosuppressive therapy than other organs, and the liver allograft is spontaneously accepted in mice (269). The mechanisms for the distinctive liver tolerance in humans and for the spontaneous liver tolerance in mice include apoptotis of alloreactive recipient cells (270), inability of the recipient dendritic cells to provide costimulatory signals as a consequence of the donor/recipient leukocyte chimerism (271), and the presence of T regulatory cells that suppress the alloimmune response (272).

In a recent study, Ma et al. (15) demonstrated the negative role of TLR9 signaling in a mouse model of liver transplantation. Ma et al. (15) showed that administration of CpG-DNA to transplant recipients 7 days after liver transplantation induced acute inflammatory infiltrate and hepatocyte damage in the liver allograft, acute allograft rejection and decreased survival of animals. Consistent with the induction of inflammatory cytokines mediated by TLR9, the acute allograft rejection was associated with increased activation of NF-κB. In addition, administration of CpG ODN that activates TLR9, breached the liver allograft tolerance and dramatically induced Th1-immune response.

2. Specific aims

To gain insight into the downstream effects of Toll-like receptors in alcohol-induced liver injury, non-alcoholic steatohepatitis and Toll-like receptor 9 –associated liver injury, we proposed the following specific aims:

A. To elucidate the role of common allelic variants in genes involved in Toll-like receptor 4-mediated liver injury in genetic susceptibility to alcoholic liver cirrhosis.

B. To determine the cell-specific effect of the Interferon regulatory factor 3 (IRF3) in the pathogenesis of alcohol-induced liver injury

C. To investigate the role of probiotics in modulation of liver inflammation, injury and fibrosis in the pathogenesis of non-alcoholic steatohepatitis

D. To assess the role of Type I interferons in the pathogenesis of Toll-like receptor 9 –induced liver injury

3. Methods

3.1. General methods

Nucleic acid and protein isolation from human and animal cells and tissues
Restriction analysis of DNA
Reverse transcription of total RNA (generation of cDNA)
Polymerase chain reaction, quantitative real-time polymerase chain reaction
DNA electrophoresis on agarose and polyacrylamide gel
Enzyme-linked immunoabsorbent assay (ELISA)
SDS-PAGE protein electrophoresis and protein immunobloting (Western blotting)
Biochemical analysis of alanine aminotransferase and lactate dehydrogenase activity
Cell culture techniques
Flow cytometry
Immunohistochemistry
Experimental work with small laboratory animals, including *in situ* liver perfusion and cell isolation
Descriptive statistics, parametric and non-parametric tests of statistical hypotheses, power calculation, regression analysis
Statistical methods in genetics and in genetic epidemiology

3.2. Specific methods

3.2.1. Methods related to genetic susceptibility to alcoholic liver cirrhosis

DNA genotyping. Genomic DNA was isolated from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). Single nucleotide allelic variants were determined by polymerase chain reaction (PCR) followed by restriction fragment length (RFLP) analysis of the products. Genotyping of the *IL1RN* variable number of tandem repeats (VNTR) locus was performed by a PCR-based fragment length polymorphism method. Restriction endonucleases were purchased from Fermentas (Fermentas UAB, Vilnius,

Lithuania). After testing for Hardy-Weinberg equilibrium (HWE), allele frequencies were checked for consistency with data from the population of European ancestry [Utah Residents with Northern and Western European Ancestry (CEU)] from the HapMap database (www.hapmap.org).

Statistical analysis. Two-sided power calculations at p=0.05 for 80% statistical power were performed using the DSTPLAN software (http:// linkage.rockefeller.edu/soft). When the odds ratio (OR) of a polymorphism was assumed to be 2, the required sample size was 100 cases and 180 controls for the polymorphism with frequency of 0.5 (*CD14* –159C/T). When the OR was assumed to be 4, the same sample size was sufficient to detect a true effect of a polymorphism with frequency of 0.03 (*TNFA* –238G/A). HWE of alleles at individual loci was evaluated using the program HWE (http://linkage.rockefeller.edu/soft). Haplotype frequencies for pairs of alleles and linkage disequilibrium (LD) coefficients $D'=D/_{Dmin\ or\ max}$ and r^2 were calculated using the Arlequin software (273). Age and median alcohol consumption between the groups was compared using the Mann-Whitney test. Male to female ratios were compared using the Fisher exact test. All association analyses were performed by logistic regression analysis using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Where applicable, logistic regression analysis adjusted for age was performed.

3.2.2. Methods related to the role of IRF3 in the pathogenesis of alcohol-induced liver injury

Animal and experimental protocol. Six to eight-week-old, female C57Bl/6 wild-type, *Irf3*-deficient (IRF3-KO) and Type I interferon α/β receptor 1-deficient (IFNAR1-KO) mice (kind gift of Jonathan Sprent, Scripps Research Institute, La Jolla, CA), were employed. Chimeric mice were generated by transplanting wild-type (C57Bl/6) bone marrow into irradiated WT mice (WT/WT-BM) to *Irf3* deficient mice (IRF3-KO/WT-BM), or by transplanting IRF3 deficient bone marrow into WT mice (WT/IRF3KO-BM). Some animals were fed with the Lieber-DeCarli diet (Dyets, Inc., Bethlehem, PA) with 5% (vol/vol) ethanol (36% ethanol-derived calories) for 4 weeks; pair-fed control mice matched the alcohol-derived calories with dextran-maltose. Serum was stored at -80°C. Livers were snap-frozen in liquid nitrogen for proteins, or stored in RNAlater (Qiagen GmbH, Hilden, Germany) for RNA extraction, or fixed in 10% neutral-buffered formalin for histopathological analysis.

Bone marrow transplantation protocol. Female mice were lethally irradiated with 900 rads (cGy) from a Cesium irradiator (Gammacell 40, Atomic Energy of Canada), and 4 hours later transplanted with 5 million male bone marrow cells via a single tail vein injection. Marrow was collected from the tibia and femur of donor 8-week old male mice by flushing with a 25g needle and passed through a cell strainer to remove clumps. Transplanted mice were housed in microisolator cages and placed on antibiotic water (Sulfamethoxazole/Trimethoprim, Hi-Tech Pharmacal Inc.) until engraftment is complete at 4-6 weeks. Engraftment was confirmed by PCR genotyping of blood cells and the chimeric mice were used at 6 weeks post-transplantation. In pilot experiments mice received GFP expressing total bone marrow from C57BL/6J-beta-actin-EGFP (Jackson laboratories, Bar Harbor, Maine, USA) mice and engraftment was monitored by FACS analysis of peripheral blood collected from the tail vein. The survival rates for all transplanted mice were >95% with excellent engraftment, e.g., >90% of the peripheral blood is of donor origin at 6 weeks post-transplant by FACS (eGFP donors) and only donor alleles were detected by PCR genotyping of blood cells (i.e., IRF3 KO donors).

Biochemical assays. Serum alanine aminotransferase (ALT) was determined using a kinetic method (D-Tek LLC., Bensalem, PA). Liver triglyceride levels were assessed using the L-Type Triglyceride H kit (Wako Chemicals USA Inc., VA).

Cytokine measurement. Mouse IL-1β ELISA kit was purchased from R&D (R&D systems, Inc., Minneapolis, MN), mouse and human TNF-α, IL-1β and IL-10 kits from BD Bioscience (BD Biosciences, San Jose, CA) and mouse IFN-β kit from PBL (PBL interferon source, Piscataway, NJ).

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). SybrGreen-based real-time quantitative polymerase chain reaction was performed using the iCycler (Bio-Rad Laboratories Inc., Hercules, CA).

Protein quantification. Whole-cell lysates were extracted from liver, as described (274). Equal amounts of proteins were separated on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane. Interleukin-10 was detected by western blot and immunostaining with specific primary antibody, followed by horseradish peroxidase-labeled secondary antibody (both from Santa Cruz Biotechnology, Santa Cruz, CA). The specific immunoreactive bands of interest were detected by chemiluminescence (Amersham, Piscataway, NJ), and quantified by densitometric analysis.

Histopathological analysis. Sections of formalin-fixed livers were stained with hematoxylin and eosin or by Oil-red-O and analyzed by microscopy.

Isolation of hepatocytes and liver mononuclear cells. Animals received anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg); the livers were perfused with saline solution for 5 minutes followed by *in vivo* digestion with Liberase blendzyme 3 (Roche Diagnostics GmbH, Mannheim, Germany) for 5 minutes at 37°C. The hepatocytes were separated by centrifugation at slow speed (350 g), liver mononuclear cells (LMNCs) were purified by centrifugation in Percoll gradient.

Phenotype analysis by flow cytometry. Cells were washed in PBS and incubated with anti-albumin (FITC), anti-F4/80 (PE), anti-CD68 (FITC), anti-GFAP (Alexa 488) antibodies for 30 minutes on ice. After incubation, cells were washed with PBS, fixed in paraformaldehyde and analyzed by flow cytometry. Some cells were permeabilized and stained for intracellular IL-10 using the anti-IL10 (Alexa 488) antibody. All antibodies were from eBioscience (eBioscience, Inc., San Diego, CA).

Isolation of human peripheral blood mononuclear cells. Human peripheral blood mononuclear cells (PBMCs) were separated from blood of healthy volunteers by centrifugation in Ficoll gradient.

In vitro experiments. Primary hepatocytes and LMNCs were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% insulin, transferrin, selenium solution. Primary hepatocytes were seeded in 6-well collagen-coated plates, LMNCs (10^6/insert) were plated in cell-culture inserts with pore diameter 0.4 µm (Becton Dickinson Labware, Bedford, MA). Hepatocytes, LMNCs or co-cultures of hepatocytes with LMNCs were stimulated with LPS (Sigma, St. Louis, MO). IFN-β, IL-10 and TNF-α were measured in supernatants using ELISA. RAW264.7 macrophages were stimulated with LPS, recombinant mouse IFN-α2a (eBioscience, San Diego, CA), recombinant mouse IL-10 (Peprotech Inc., Rocky Hill, NJ) or with anti-mouse IL-10 receptor antibody (Biolegend, San Diego, CA). Human PBMCs were stimulated with LPS, recombinant human IFN-α (PBL, Piscataway, NJ), recombinant IL-10 (Ebioscience, San Diego, CA) or IL-10 receptor antibody (R&D systems, Inc., Minneapolis, MN).

Hepatocyte cytotoxicity assay. Lactate dehydrogenase (LDH) release from the hepatocyte into the culture supernatants was measured using the LDH-cytotoxicity assay kit (Abcam, Cambridge, MA), and normalized to total LDH (determined after treatment of cells with detergent-based lysis solution).

3.2.3. Methods related to the role of probiotics in the pathogenesis of non-alcoholic steatohepatitis

Animal and experimental protocol. C57Bl/6 mice were fed a methionine-choline-deficient (MCD) diet or a methionine-choline-supplemented (MCS) diet (Dyets, Inc. (Bethlehem, PA); the latter control diet was composed of MCD diet supplemented with L-methionine (1.7 g/kg) and choline bitartrate (14.48 g/kg). The mice were fed these diets for a total period of 10 weeks; there were 6-8 mice per experimental group. After a week of adaptation to the new diets, the MCD diet-fed mice were divided into two groups, with one group having its water supply replaced by water containing VSL#3 (VSL Pharmateuticals, Ft. Lauderdale, FL) for the remaining 9 weeks of the experimental feeding. One packet of VSL#3 (450 billion colonies/packet) was mixed in 1L of water and provided to mice instead of drinking water freshly made daily. In pilot experiments we were able to isolate viable VSL#3 bacteria from stool in VSL#3–fed mice; no pathogenic bacteria were isolated from this water. At the end of the 10 week-feeding period some animals were challenged with LPS (0.5mg/kg body weight, i.p) or comparable volumes of saline injected as control.
Histopathological analysis. Sections of formalin-fixed, paraffin-embedded livers were stained with hematoxylin and eosin to assess for histologic features of steatohepatitis, and sirius red stain and trichrome stain (Masson's method) to evaluate for hepatic collagen deposition and fibrosis. The liver sections were also subject to immunohistochemical staining (anti-α–smooth muscle actin antibodies from Abcam, Cambridge, MA).
Protein quantification. Equal amounts of liver protein extract from different stimulation groups were separated on a 10% polyacrylamide gel, and transferred to a nitrocellulose membrane. The proteins of interest were detected by western blot and immunostaining with specific primary antibodies, followed by horseradish peroxidase (HRP)-labeled secondary antibodies (anti-Collagen and anti-α-SMA from Abcam, Cambridge, MA, secondary-HRP labeled antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). The specific immunoreactive bands of interest were detected by chemiluminescence (Amersham, Piscataway, NJ), and quantified by densitometric analysis.
Electrophoretic Gel Mobility Shift Assay was performed using equal amounts (5 μg) of nuclear protein and a 32P-labeled consensus oligonucleotide specific for NF-κB (Promega, Madison, WI) or PPRE (Santa Cruz Biotechnology, Santa Cruz, CA). For dissection of PPAR complex composition, nuclear proteins (5 μg) were pre-incubated with 2μl of specific

antibodies (anti-PPARα, anti-PPARγ and anti-RXR, all from Santa Cruz Biotechnology) for 1 hour, followed by 32P-labeled consensus oligonucleotide specific for PPRE. In every assay, a 20-fold excess of specific unlabeled double-stranded specific probe was added to a separate reaction mixture for cold competition assay. Protein-DNA complexes were subsequently resolved in polyacrylamide gels; the gels were then dried and exposed to autoradiographic films at –80°C.

3.2.4. Methods related to the role of Type I interferons the pathogenesis of TLR9-associated liver injury

Animals and experimental protocol. The B6.129F2 and C57Bl/6 wild-type (WT) mice were purchased from Jackson Laboratory. *Irf7*-deficient (IRF7-/-) mice on B6.129F2 background were provided by Tadagatsu Tanaguichi (Department of Immunology, Graduate School of Medicine and Faculty of Medicine, University of Tokyo, Tokyo, Japan) and type I interferon α/β receptor 1-deficient (IFNAR1-/-) mice on the C57Bl/6 background were the kind gift of Jonathan Sprent (Scripps Research Institute, La Jolla, CA). All animals were 6–8 weeks old. We employed a previously described model of TLR9-associated liver injury induced by administration of TLR9 and TLR2 ligands. After acclimatization, WT, IRF7-/- and IFNAR1-/- mice were injected intraperitoneally (i.p.) with saline or the combination of 2.5 mg/kg unmethylated DNA rich in cytidine-phosphate-guanosine (CpG, ODN1826 murine TLR9 ligand; InvivoGen, San Diego, CA), and 5 mg/kg lipoteichoic acid (LTA, from Staphylococcus aureus; Sigma, Saint Louis, MO). Three days after the above priming stimulus, the mice were injected i.p. with either saline or 0.5 mg/kg lipopolysaccharide (LPS, from Escherichia coli 0111:B4, Sigma, St. Louis, MO) and sacrificed as indicated. Some C57Bl/6 WT mice received a single i.p. injection of 100,000 IU human pegylated interferon alpha-2b (pegIFNα2, Pegintron, Schering, Kenilworth, NJ) two hours prior to LPS. Others were pretreated with recombinant human interleukin-1 receptor antagonist (IL-1ra) 25 mg/kg i.p. every six hours (Anakinra, Amgen, Thousand Oaks, CA) for 24 hours before CpG+LTA, and the treatment with IL-1ra was ongoing until sacrifice. Serum was separated by centrifugation. Livers were snap frozen, stored in RNAlater (Qiagen GmbH, Hilden, Germany) or fixed in 10% formalin. ALT was quantified by biochemical assay (D-Tek Analytical Laboratories Inc, San Diego, CA).

Histopathology analysis. Sections of formalin-fixed, paraffin-embedded livers were stained with hematoxylin and eosin (H&E), and assessed for inflammatory infiltrate; area of inflammatory infiltrates was calculated with Microsuite (Olympus Soft Imaging Solution GmbH, Munster, Germany) image analysis software in 20 high power fields.

Isolation of hepatocytes and liver mononuclear cells. Animals received anesthesia with ketamine and xylazine; the livers were perfused with saline solution followed by in vivo digestion, as we previously described. The hepatocytes and liver mononuclear cells (LMNCs) were purified by centrifugation at slow speed (350g) and in Percoll gradient, respectively.

Phenotype analysis by flow cytometry. Cells were washed in PBS and incubated with anti-CD68 (FITC), anti-CD11c (FITC) or anti-PDCA1 (Alexa Fluor 647) antibodies for 30 minutes on ice. After incubation, cells were washed with PBS, fixed in paraformaldehyde and analyzed by flow cytometry. All antibodies were from eBioscience (eBioscience, Inc., San Diego, CA).

In vitro cell culture. Primary hepatocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% insulin, transferrin, and selenium supplement on collagen-coated plates (Becton Dickinson Labware, Bedford, MA). Primary LMNCs and murine lymph node endothelial cells SVEC4-10 (ATCC No. CRL-2181) were cultured in DMEM with 10% FBS. Hepatocytes were treated with recombinant murine IFN-α2b (500 IU/mL, eBioscience, San Diego, CA), murine IL-1ra (100 pg/mL, R&D Systems, Minneapolis, MN), murine IL-1β (100 IU/mL, Peprotech Inc., Rocky Hill, NJ) or murine TNF-α (0-100 ng/mL, Peprotech). LMNCs were treated with mouse IFN-α2b or LPS (100 ng/mL, Sigma, St. Louis, MO); SVEC4-10 cells were treated with recombinant murine IFN-α2b.

Hepatocyte cytotoxicity assay. Lactate dehydrogenase (LDH) release from the hepatocyte into the culture supernatants was measured using the LDH-cytotoxicity assay kit (Abcam, Cambridge, MA), and normalized to total LDH (determined after treatment of cells with detergent-based lysis solution).

Cytokine and chemokine measurement. Interleukin 1β and tumor necrosis factor alpha (TNF-α) were measured using ELISA from BD Biosciences (BD Biosciences, San Jose, CA); IL-1 receptor antagonist (IL-1ra) and monocyte chemotactic protein 1 (MCP-1) ELISAs were from R&D (R&D Systems, Minneapolis, MN).

4. Results and discussion

We assessed the role of downstream signals induced by Toll-like receptors in the context of alcoholic liver disease and non-alcoholic steatohepatitis, and in liver inflammation and damage mediated by TLR9 signaling. We used a broad spectrum of approaches, including population-based genetic testing, *in vivo* animal models and *in vitro* methods to identify pathogenic mechanisms that could be of potential use in diagnosis and therapy of liver diseases.

4.1. Specific aim A - The role of allelic variants in TLR-induced signaling in genetic susceptibility to alcoholic liver cirrhosis

Enclosure 1: **Petrasek J**, Hubacek JA, Stickel F, Sperl J, Berg T, Ruf E, et al. Do common genetic variants in endotoxin signaling pathway contribute to predisposition to alcoholic liver cirrhosis? Clin Chem Lab Med 2009;47:398-404.

The TLR4 recognizes LPS and induces inflammatory cytokines, predominantly via the activation of the transcription factors NF-κB and IRF-3. In addition, interaction of TLR4 with NADPH oxidase is involved in LPS-mediated generation of reactive oxygen species, which contribute to NF-κB activation and further increase the production of inflammatory cytokines. To define the importance of genetic variations in TLR4-dependent inflammatory signaling and oxidation stress, we performed a multicentric allelic association study. Using a candidate-gene strategy, we investigated functional allelic variants in genes involved in LPS recognition (*LBP, CD14, BPI, TLR4*), inflammatory signaling (*TNF-A, IL-1B, IL-1RN*) and in modulation of oxidative stress (*SOD2, UCP2*).

4.1.1. Association of allelic variants involved in TLR4-mediated inflammatory signaling with alcoholic liver cirrhosis

In the pilot cohort involving 100 Czech patients with ALC and 180 Czech population controls, we investigated the frequency of allelic variants in the genes for *LBP, CD14, BPI,*

TLR4, *TNF-A, IL-1B* and *IL-1RN*. The genotype frequencies in the control individuals were in HWE, suggesting a normal distribution of genotypes in the population under study, and were consistent with the reference data from Utah residents with a Northern and Western European ancestry (CEU) population from the HapMap database (275). The *TNF-A* -863 locus, which showed a deviation from HWE due to an excessive frequency of homozygotes for the minor A allele, was *a priori* excluded from the association analysis.

We observed a complete LD between the *TLR4* +896 and the *TLR4* +1196 loci (D’ > 0.9, $r^2 = 0.7$) and an absolute LD between the *IL-1B* -511 and the *IL-1B* -31 loci (D’ = 1, $r^2 = 1$). The LD between the *IL-1B* -31 and the *IL-1RN* was weak (D’ < 0.5). Alleles in the *TNF-A* -863 and *TNF-A* -238 and in the *LBP* +1306 and *LBP* +645 demonstrated a random segregation (Table 1). The complete LD between the loci *TLR4* +896 and *TLR4* +1196 and the absolute LD between loci *IL-1B* -31 and *IL-1B* precluded haplotypic analysis due to oversaturation of the statistical model in which inclusion of both loci would be redundant. Similarly, the absence of LD between the loci *LBP* +1306 and *BPI* +645 suggested a random segregation of alleles which was not suitable for haplotype analysis. On the other hand, the low strength of LD between the *IL-1B* -31 and the *IL-1RN* loci permits an additive effect of the proinflammatory alleles *IL-1B* -31T and *IL-1RN**2 with a larger effect size than would be expected from either of the alleles alone (276). Therefore, in addition to the single locus analysis, we performed an association analysis of the [*IL-1B* -31; *IL-1RN*] haplotype.

Table 1. *Haplotype frequencies and linkage disequilibrium coefficients for pairs of syntenic loci.*

	Haplotype						**Linkage disequilibrium**			
Locus	**1-1**	**1-2**	**2-1**	**2-2**	**1-3.4.5**	**2-3.4.5**	***D'***	r^2	χ^2	***P***
Patients (n = 100)										
TLR4 +896/*TLR4* +1196	0.909	0.01	0.005	0.075			0.93	0.81	162.3	0.0001
LBP +1306/*BPI* +645	0.049	0.056	0.476	0.419			-0.11	0.01	0.33	0.56
TNF-A -863/*TNF-A* -238	0.815	0.045	0.139	0.00003	-	-	-0.99**	0.01	1.52	0.22
IL-1B -31/*IL-1B* -511	0.365	0	0	0.635	-	-	1.00	1.00	200	0.0001
IL-1B -31/*IL-1* RN*	0.174	0.190	0.485	0.145	0	0.005	0.32	0.09	9.04	0.0001
Controls (n = 180)										
TLR4 +896/*TLR4* +1196	0.941	0	0.016	0.041			0.99	0.70	252.6	0.0001
LBP +1306/*BPI* +645	0.054	0.069	0.492	0.393			-0.13	0.01	0.89	0.35

TNF-A -863/*TNF-A* -238	0.806	0.028	0.161	0.006	-	-	0.01	0.01	0.0001	0.99
IL-1B -31/*IL-1B* -511	0.355	0	0	0.644	-	-	1.00	1.00	359	0.0001
IL-1B -31/*IL-1* RN*	0.157	0.192	0.534	0.097	0.006	0.013	0.47	0.16	30.44	0.0001

*) PCR-RFLP products of the *IL-1RN* VNTR variants are coded as follows: allele 1 = 4 repeats (size 442-bp), allele 2 = 2 repeats (280-bp), allele 3 = 5 repeats (528-bp), allele 4 = 3 repeats (356-bp), allele 5 = 6 repeats (614-bp). The rare alleles 3, 4 and 5, which represent less than 5% of allelic variants of *IL-1RN*, were pooled for statistical analysis.

**) The D' value for the haplotype *TNF-A* -863/*TNF-A* -238 is an inflation artifact due to the low frequency of the 2-2 haplotype.

4.1.1.1 Haplotype analysis

We observed that patients with ALC showed a significantly higher frequency of the [*IL-1RN**2/*2; *IL-1B* -31T+] diplotype, consisting of alleles that increase the production of IL-1β *in vitro* (239, 240), and that the [*IL-1RN**2/*2; *IL-1B* -31T+] diplotype was significantly associated with ALC (odds ratio for ALC = 9.45, 95% confidence interval 1.96 – 45.7, p = 0.006) (Table 4 in Enclosure 1). The association remained significant even after correction for testing of multiple statistical hypotheses ($P_{corrected}$ = 0.048). This finding would suggest that a genetically determined increase in IL-1β signaling contributes to the increased susceptibility to alcoholic liver disease. Indeed, *in vitro* studies have shown that IL-1β increases the susceptibility of hepatocytes to cytotoxicity induced by TNF-α, a principal cytokine critically involved in the pathogenesis of ALD (215, 277, 278).

4.1.1.2 Single locus analysis

Analysis of individual genetic variants *LBP* c.1306T/C, *BPI* c.645A/G, *CD14* -159C/T, *TLR4* c.896A/G, *TLR4* c.1196C/T, *TNF-A* -238G/A, *IL-1B* -511T/C, *IL-1B* -31C/T and *IL-1RN* VNTR in our cohort did not show an association with ALC (Table 3 in Enclosure 1 and Table 2).

Table 2. *Genotype frequencies in 100 Czech patients with ALC and 180 healthy controls (Extension of Table 3 from Enclosure 1).*

		Patients		Controls		Association with alcoholic cirrhosis		
Lokus	Genotype	n = 100	%	n = 180	%	χ^2	*P*	OR (CI 95%)
LBP +1306	T/T	79	79.0	139	0.77			1.00
	C/T	21	21.0	41	0.23	0.10	0.75	0.91 (0.52 - 1.59)
	C/C	0	0.0	0	0.00	-	-	-
BPI +645	A/A	29	0.29	52	0.29			1.00
	A/G	47	0.47	93	0.52	0.26	0.61	1.09 (0.77-1.55
	G/G	24	0.24	35	0.19	0.80	0.37	1.31 (0.73 - 2.36)

4.1.1.3 Validation of positive findings

The current requirements for the methodology of allelic association studies (279), led us to validate the association of the [*IL-1RN**2/*2; *IL-1B* -31T+] diplotype with ALC in additional cohorts. Unfortunately, we failed to confirm the association in an extended cohort of 198 Czech patients and 370 Czech controls, and in a second cohort of 173 German patients and 331 German controls.

To achieve the highest possible power to detect a true association with ALC, we recruited an alternative control group of 109 German heavy drinkers without liver disease. These control individuals, who did not develop ALC in spite of heavy alcohol consumption (hypernormal controls), are expected to have a low frequency of alleles predisposing to ALC and a higher frequency of alleles that protect from the disease. In spite of including hypernormal controls, we did not find an association of the [*IL-1RN**2/*2; *IL-1B* -31T+] diplotype with ALC (Table 4 in Enclosure 1). It is therefore likely that the initial positive finding in the pilot group of 100 patients and 180 controls resulted in an overestimation of the true effect size, which was identified as a Type-I error (false positivity) in subsequent validation steps.

4.1.2. Allelic variants in genes involved in the protection against oxidative stress

We investigated allelic variants in the genes for SOD2 and UCP2 in 158 German patients with ALC and 400 population controls. The genotype frequencies were in HWE with the exception of the locus *UCP2* +164, which showed a deviation from HWE due to an

excessive frequency of homozygotes for the rare A allele, and was therefore excluded from the association analysis. We observed a weak LD between the *UCP2* -866 and the *UCP2* ins/del 45bp 3'UTR (D' < 0.5, $r^2 = 0.12$) (Table 3).

Table 3. *Haplotype frequencies and linkage disequilibrium coefficients for pairs of syntenic loci.*

Locus	Haplotype				Linkage disequilibrium			
	1-1	**1-2**	**2-1**	**2-2**	***D'***	r^2	χ^2	***P***
Patients (n = 158)								
UCP2 -866/*UCP2* ins/del 45bp 3´UTR	0.518	0.069	0.170	0.243	0.62	0.25	50.0	0.0001
Controls (n = 400)								
UCP2 -866/*UCP2* ins/del 45bp 3´UTR	0.485	0.116	0.193	0.206	0.40	0.12	87.2	0.0001

Analysis of individual genetic variants *SOD2* c.47C/T, *UCP2* -866G/A and *UCP2* ins/del 45bp 3'UTR did not show an association with ALC (Table 4). Similarly, we did not find any difference in distribution of the [*UCP2* -866; *UCP2* ins/del 45bp 3´UTR] haplotypes between patienst and controls (global *P* value for difference = 0.19), supporting no association with ALC.

Table 4. *Genotype frequencies in 158 Czech patients with ALC and 400 healthy controls*

Locus	Genotype	Patients		Controls		Association with alcoholic cirrhosis		
		n = 158	**%**	**n = 400**	**%***	χ^2	***P***	**OR (CI 95%)**
SOD2 +47	C/C	32	0.20	97	0.24			1.00
	C/T	95	0.60	199	0.50	0.13	0.72	0.95 (0.73 - 1.24)
	T/T	31	0.20	104	0.26	2.51	0.11	0.69 (0.44 - 1.09)
UCP2 -866	G/G	51	0.32	133	0.33			1.00
	G/A	82	0.52	206	0.51	0.10	0.75	1.04 (0.80 - 1.36)
	A/A	25	0.16	62	0.15	0.01	0.92	1.02 (0.64 - 1.70)
UCP2 ins/del 45bp 3´UTR	ins/ins	75	0.47	195	0.49			1.00
	ins/del	68	0.43	154	0.38	0.09	0.76	0.92 (0.72 - 1.27)
	del/del	16	0.10	51	0.13	0.74	0.39	0.77 (0.43 - 1.40)

4.1.3. Association of the *TNF-A* -238G/A allelic variant with alcoholic liver cirrhosis (meta-analysis)

In our study, we did not observe any significant association of the *TNF-A* -238A allele with ALC, contrary to previously published studies that suggested an association of this allele with alcoholic steatohepatitis (280) and cirrhosis (281). On the other hand, our results were compatible with studies that demonstrating no association with ALD (282-285). This controversy is likely due to a low frequency of the *TNF-A* -238A allele (~3%) in the population, low power of individual studies to detect a true association of the risk allele with ALD, and lack of validation cohorts that would confirm the initial positive findings (discussed in (23, 279)).

Our group was asked by dr. M. Marcos, at the University of Salamanca, Spain, to submit the results of the *TNF-A* -238G/A genotyping to a meta-analysis based on data from a total of 11 studies (Marcos et al., Am J Epidemiol 2009;170(8):948-56, our contribution acknowledged). Concerning the *TNF-A* -238G/A polymorphism, the authors found a significant association of the *TNF-A* -238A allele and the risk of alcoholic liver cirrhosis (odds ratio = 1.47, 95% confidence interval: 1.05 - 2.07) (286). In spite of a significant statistical association of the *TNF-A* -238G/A with ALD, the low odds ratio (relative risk) suggested its weak biological effect in the pathogenesis of ALC.

4.1.4. Discussion

In our study, we included only those cytokines whose role in the pathogenesis of alcohol induced liver injury was proved in animal models and those allelic variants whose impact on the expression of the respective gene or the function of the respective protein was described in *in vitro* studies. However, in contrast to published reports (280-284, 287-290), we found no association between any of the functionally relevant polymorphisms and the risk of alcoholic liver cirrhosis. The only consistent association of an allelic variant involved in TLR-dependent signaling with ALC was calculated by Marcos et al. (286) for the *TNF-A* -238 locus after pooling more than 800 patients and 1000 controls from 11 different studies, including our data (25); however, the low relative risk conferred by this allele suggested its minor biological importance. Taken together, our data suggest a limited value of allelic association studies in ALD. This notion has two aspects that relate to the methodology of

genetic association studies, and to the relative importance of allelic variants in multifactorial diseases with strong exogenous causal factors.

The first explanation refers to the discrepancy between individual single-center studies that often fail to conform to the currently accepted guidelines for the design of genotype/phenotype association studies (279, 291). The prerequisites for the design of allelic association studies include a logical rationale for the chosen candidate genes, coherent hypothesis based on the functional significance of the studied genetic variants, inclusion of cases and controls with comparable baseline characteristics, assurance of the reliability of genetic testing by calculating the HWE, correction for testing of multiple statistical hypotheses and a sufficient statistical power (discussed in detail in our recently published work (23, 292, 293)).

In particular, insufficient statistical power to detect a true allelic association represents the most obvious explanation for the variability of findings in different studies of the same condition. The key quality determinant of an association study is the sample size (279), which should be determined by the power calculation in the study-designing phase. Results obtained from inadequately powered studies tend to have a decreased probability of detecting a true effect of a polymorphism due to the type II error (false negativity). Moreover, for any choice of significance level, the proportion of false-positive results among all positive results (type I error) is greatly increased as power decreases (294). Our calculation revealed that none of the studies reporting an association of polymorphisms in TLR4-induced signaling with ALD complied with the current demands for 80% power (280-284, 287-290). Moreover, none of the studies included an independent validation cohort, which should be implemented particularly in small studies that are likely to overestimate the true effect size (295).

The second explanation refers to the limited biological role of allelic variants in the pathogenesis of multifactorial diseases with a strong exogenous component that induces a broad spectrum of pathological interactions. The typical effect sizes of individual genetic variants for complex diseases are modest, pertaining to odds ratios of 1.2 – 1.6 (296, 297). Using a multivariate regression modeling, we demonstrated that the relative contribution of allelic variants to a complex clinical outcome, such as kidney transplant rejection, did not exceed 1% and that the majority of risk was attributable to much stronger clinical and demographic determinants (276). That would suggest that the genetic component of complex diseases relies upon multiple genetic loci with a small relative contribution. Indeed, it has been calculated that that the combination of a few genetic variants (10 to 20) at multiple loci, each with a small effect size (odds ratio of about 1.5), may account for a substantial

proportion of the population attributable fraction for many common diseases (298). Unfortunately, the calculated sample size that would enable association analysis of multiple allelic variants with small effect size encompasses between 50000 – 100000 individuals to answer questions even of modest complexity (296). Clearly, this requirement is beyond the sample size of the two recent meta-analyses on genetic predisposition for alcoholic liver disease (233, 286), and even the single largest general-purpose observational cohorts and biobanks (299, 300) would be challenged to meet these numbers.

A novel approach to identification of candidate genes involved in the pathogenesis of complex diseases is represented by the genome-wide association studies (GWAS) (301). Among the GWAS publications, twelve are concerned with hepatological conditions, including NASH. A recent GWAS demonstrated a genetic association of the rs738409 locus in the *PNPLA3* gene, encoding adiponutrin, with NASH (302). Prompted by these results, another research team has currently found a strong association of the rs738409 locus with alcoholic liver cirrhosis in Mestizo individuals in Mexico City (303). However, the authors restricted the analysis to the genomic region surrounding the *PNPLA3* locus and did not analyze any allelic variants relevant to the signaling mediated by Toll-like receptors.

The original idea behind the allelic association studies was that testing of candidate alleles would enable a reliable assessment of genetically determined individual risk for a particular disease and enable an early intervention. From that perspective, our data and studies of others (23, 280-282, 286, 288, 303-305) available so far are disappointing and can hardly be used for any predictive modeling.

In conclusion, our results imply that although there is a little doubt that cytokine-mediated immune reactions play a role in the pathogenesis of ALC, hereditary susceptibility caused by variants in key genes involved in TLR4-mediated liver injury seems to be low, or at least such is the case in central European population (23). It remains possible that genetic predisposition to alcoholic liver cirrhosis is determined by multiple polymorphisms with a low individual contribution to the phenotype that cannot be assessed in allelic association studies. Alternatively, other as yet unidentified polymorphisms may significantly affect the risk of alcoholic liver cirrhosis. Therefore, identification of such polymorphisms warrants future large-scale, multicentric studies that will be fully compliant with the currently accepted standards for genetic association studies.

4.2. Specific aim B - The cell-specific role of the interferon regulatory factor (IRF3) in the pathogenesis of alcoholic liver disease

Enclosure 2: **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, et al. Hepatocyte-specific IRF3 and Type I interferons are protective in alcohol-induced liver injury in mice via cross-talk with macrophages. 2010; Manuscript submitted.

TLR4 recognizes LPS and activates two signaling pathways by utilizing the adaptor molecules MyD88 and TRIF, respectively. Previously, it was showed that MyD88 is dispensable in ALD (182). In addition to induction of inflammatory cytokines via NF-κB, MyD88-independent activation of TLR4 triggers production of Type I IFNs, which is largely dependent on activation of intracellular pathways involving the IRF3 (32).

4.2.1. Summary of results

To study the role of IRF3 in the pathogenesis of alcohol-induced liver injury, we fed wild-type mice (WT), *Irf3*-deficient mice (IRF3KO), WT mice with transplanted WT bone marrow (WT/WT-BM), WT mice with *Irf3*-KO transplanted bone marrow (WT/IRF3KO-BM) and IRF3KO mice with transplanted WT bone marrow (IRF3KO/WT-BM) with Lieber-DeCarli diet (5% v/v ethanol) or control diet for 4 weeks.

Alcohol feeding resulted in liver injury, steatosis and induction of inflammatory cytokines in WT mice but not in IRF3KO mice. As this finding suggested that IRF3 is involved in the pathogenesis of ALD, we tested the selective contribution of Irf3 in BM-derived cells (WT/IRF3KO-BM mice) and in hepatocytes (IRF3KO/WT-BM mice). We observed that the WT/IRF3KO-BM chimeras showed partially ameliorated alcohol-induced liver injury and induction of inflammatory cytokines, compared to WT/WT-BM controls, supporting a proinflammatory role of IRF3 in BM-derived cells in ALD (184).

In contrast, IRF3KO/WT-BM chimeras fed with alcohol showed increased liver injury, steatosis and serum inflammatory cytokines, but a significantly decreased expression of Type I IFNs and IL-10, an anti-inflammatory cytokine, compared to WT mice. Co-cultures of primary hepatocytes and primary mononuclear cells isolated from WT, *Irf3* and Type I

IFN receptor α/β–deficient mice demonstrated that hepatocyte-derived IFN-β, dependent of IRF3, induces anti-inflammatory IL-10 and suppressed TNF-α and IL-1β in liver macrophages in paracrine manner.

4.2.2. Discussion

The study on the role of IRF3 in the pathogenesis of ALD is an extension of the previous study that has demonstrated that MyD88 is dispensable for TLR4-mediated ALD (182), and that suggested a dominant role for the TRIF-dependent pathway, mediated by IRF3. Indeed, the data from the present study demonstrate a complete protection from alcohol-induced liver injury in mice with global deficiency for *Irf3*, as documented by decreased liver injury, steatosis and induction of inflammatory mediators, compared to wild-type mice (24). The results therefore confirm the hypothesis IRF3 is indispensable for the pathogenesis of ALD, and suggest that IRF3 is the key transcription factor mediating the pro-inflammatory effect of the TLR4-/TRIF-dependent signaling. In addition, the study presents several novel concepts suggesting a protective role of IRF3 in hepatocytes, mediated by Type I IFNs, and emphasizes the active role of hepatocytes in modulating the extent of the innate immune response elicited by LPS/TLR4.

4.2.2.1. The role of macrophage-specific IRF3 in induction of pro-inflammatory cytokines

Chronic ethanol abuse results in the development of steatosis, hepatitis and cirrhosis. Augmented TNF-α production by macrophages and Kupffer cells and signaling via the TNF-α receptor has been shown to be critical for these effects of chronic ethanol; however, the molecular mechanisms leading to augmented TNF-α production remain unclear. Using cell culture models and *in vivo* studies, Zhao et al. (184) demonstrated that chronic feeding with ethanol resulted in increased TNF-α transcription, which was independent of NF-κB. Using reporter assays and chromatin immunoprecipitation, they found that this increased transcription was attributable to increased IRF3 binding to and transactivation of the *TNF-A* promoter. As IRF3 is downstream from the TLR4 adaptor TRIF, Zhao et al. (184) demonstrated that macrophages from Trif-/- mice were resistant to upregulation of TNF-α transcription by ethanol *in vitro* as well as ethanol-induced steatosis and TNF-α upregulation *in vivo*.

These data were consistent with previous findings that the promoter of *TNF-A* contains an IRF3 specific transcription site (306), and with our finding that absence of IRF3 protected mice from alcohol-induced liver injury (24). Taken together, these findings suggest that the synergy of NF-κB and IRF3 plays a central role in induction of the inflammatory component seen in alcohol-induced liver diseases, and support the current concept of synergistic activation of cytokines enabled by concomitant binding of multiple transcription factors, enabling context-specific fine tuning of inflammatory response (307).

4.2.2.2. Activation of IRF3 and induction of IFN-β in hepatocytes

The exaggerated liver inflammation and injury observed in *Irf3*-deficient mice with transplanted wild-type bone marrow suggested a protective role of IRF3 in liver parenchymal cells. Given the fact that a majority of liver parenchymal cells is represented by hepatocytes, we hypothesized that the protective role of hepatocyte-specific IRF3 is mediated by IFN-β upon LPS activation.

Indeed, hepatocytes have been shown to uptake and eliminate endotoxin from portal and systemic circulation (112). A study using primary mouse cultured hepatocytes demonstrated that hepatocytes expressed TLR1 through TLR9 as well as MyD88 and MD-2 transcripts, indicating that hepatocytes express all known PAMP recognition molecules. In addition, hepatocytes stimulated with LPS showed activation of NF-κB; this activation was reduced in TLR4-mutant or null hepatocytes compared to control hepatocytes, and this defect was partially restored by adenoviral transduction of mouse TLR4 (115).

We observed that stimulation of primary mouse hepatocytes with LPS induced IFN-β in wild-type primary mouse hepatocyte cultures; *Irf3*-deficient hepatocytes did not show any induction of IFN-β (Fig. 4 in Enclosure 2). This novel finding of TLR4/IRF-3 –dependent induction of IFN-β in hepatocytes supports previous reports demonstrating the capacity of hepatocytes to produce IFN-β (225).

To ensure that the production of IFN-β from primary hepatocyte cultures is not attributable to contaminating cells, we stained the isolated primary hepatocytes with markers specific for monocytes/macrophages (CD68), hepatic stellate cells (glial fibrillary acidic protein – GFAP), hepatocytes (albumin) and cholangiocytes (cytokeratin 7). Flow cytometry analysis demonstrated more than 95% purity of primary hepatocyte cultures with less than 5% contamination with other cell types (Fig. 1)

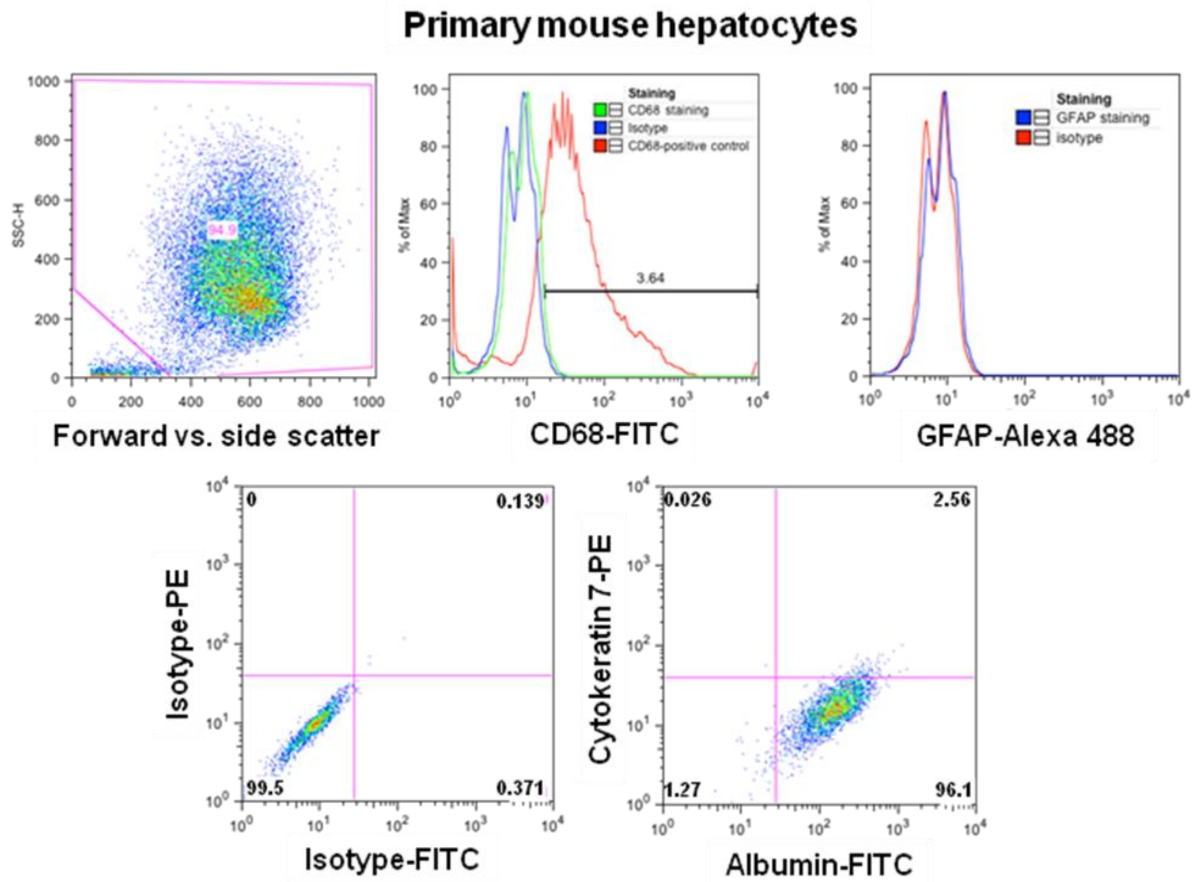


Fig. 1. Representative flow cytometry histograms and scatter plots documenting high purity of primary hepatocyte isolates. Primary hepatocytes isolated from wild-type mice were stained with antibodies against CD68-FITC (monocyte/macrophage specific) and GFAP-Alexa fluor 488 (hepatic stellate cells specific); intracellular staining for albumin-FITC (hepatocyte specific) and CK7-PE (cholangiocytes-specific) was performed after previous cell fixation and permeabilization. Gating is based on the respective isotype controls. We used primary Kupffer cells as a positive control for CD68 staining. No positive control was available for GFAP staining.

4.2.2.3. The role of crosstalk between hepatocytes and immune cells in anti-inflammatory signaling in the liver

Chronic consumption of alcohol is linked to liver steatosis and inflammation in humans as well as in experimental models. Whereas activation of TLR4-dependent pathways by gut-derived LPS and induction of inflammatory cytokines has been traditionally attributed to bone marrow-derived Kupffer cells (22), the role of crosstalk between parenchymal and non-parenchymal (bone marrow-derived cells) in alcohol-induced liver injury remains elusive.

We demonstrated that liver response to TLR ligands is a multistep process: hepatocyte-specific IRF3 drives Type I IFN induction in the liver and hepatocyte-derived Type I IFNs lead to modulation of inflammatory cytokines in BM-derived cells. We suggest that the paracrine link between hepatocyte-derived Type I IFNs and monocyte-derived inflammatory cytokines is critically regulated by the anti-inflammatory IL-10 produced by liver leukocytes. Indeed, the *IL10* gene contains a Type I IFN-responsible transcription site in its promoter that increases production of IL-10 (308).

One could also assume that liver mononuclear cells stimulate hepatocytes to produce very large amounts of IL-10, and that hepatocytes are the source of the majority of IL-10 reported in our manuscript (24). However, our additional data showed that even supraphysiological stimulation of primary hepatocytes with LPS, phorbol-myristoyl acetate, ionomycin and brefeldin A did not induce IL-10 in hepatocytes (Fig. 2). Therefore, we favor the hypothesis that the primary producing cell type of IL-10 in the liver is the mononuclear cell population.

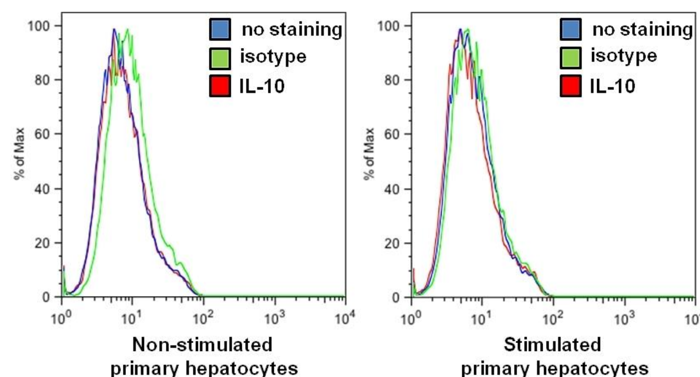


Fig. 2. Lack of IL-10 induction in primary mouse hepatocytes. Primary hepatocytes isolated from WT mice were stimulated with LPS for 12 hours, and with phorbol myristoyl acetate and ionomycin for the last 6 hours of stimulation. Brefeldin A was added for the last four hours of stimulation. Cells were washed, fixed, permeabilized, stained with anti-IL10 (Alexa fluor 488 – Fl1) and subjected to flow cytometric analysis. Representative histogram from N=3 mice is shown. The anti-IL-10 antibody was previously validated using RAW264.7 macrophages as positive controls.

Taken together, our results underscore the paracrine cross-talk between hepatocytes and liver leukocytes that fine-tunes the hepatic response to alcohol-induced injury. These findings also highlight that hepatocytes are not simply targets of inflammatory cytokines, but function also as early responders to LPS. The findings are also especially novel because they place hepatocytes at the crossroads of innate immunity regulation in the response to liver injury.

4.2.2.4 Incomplete protection from alcoholic liver injury in mice with bone marrow-specific deficiency in IRF3

Our finding that global *Irf3* deficiency protected mice from alcohol-induced liver injury, whereas *Irf3* deficiency selective for parenchymal cells aggravated liver injury suggested that the pro-inflammatory effect of IRF3 is specific for bone-marrow derived cells. That would be expected, given the fact that bone marrow-derived cells, in particular Kupffer cells, are considered the key cell population involved in inflammatory signaling in the liver (135, 136). To prove this hypothesis, we fed WT mice transplanted with *Irf3*-deficient bone marrow (WT/IRF3KO-BM) with alcohol. Surprisingly, we observed only partial protection from alcohol-induced liver injury, documented by serum ALT and histology, compared to wild-type animals (Fig. 3). On the other hand, alcohol fed WT/IRF3KO-BM mice did not show any increase in the proinflammatory cytokines TNF-α and IL-1β, compared to pair-fed animals (Fig. 3).

Based on the incomplete protection from alcohol-induced liver injury in WT/IRF3KO-BM mice, it could be speculated that BM transplantation to wild-type mice did not lead to a complete reconstitution with IRF3-KO bone marrow. However, our data do not favor this notion; instead, we demonstrate replacement of wild-type bone marrow-derived cells with cells deficient in IRF3 (Fig. 4).

In addition, the discrepancy between incomplete protection from alcohol-induced injury and complete absence of upregulation of inflammatory cytokines in WT/IRF3KO-BM mice fed with alcohol might suggest that IRF3 may have additional roles, which are independent of innate immune signaling. Indeed, such role has been recently proposed by Chattopadhyay et al. (309), who showed that IRF3 has a pro-apoptotic function independent of its transcriptional activity. Therefore, one could hypothesize that the complete protection from alcohol-induced liver injury, observed in mice globally deficient in IRF3, might be a joint result of absent induction of inflammatory cytokines in IRF3-deficient bone-marrow derived cells, and of a survival advantage of hepatocytes that lack the pro-apoptotic IRF3. This hypothesis is currently under investigation.

An alternative explanation could be that the pro-inflammatory role of IRF3 in the livers exposed to alcohol is also exerted by cells with inflammatory capacity that are not derived from bone marrow. Hepatic stellate cells, the key cell type involved in liver fibrogenesis, may be a likely candidate: a recent study demonstrated that hepatic stellate cells

express TLR4 and promptly respond to LPS stimulation *in vitro* and *in vivo* (13). However, to the best of our knowledge, no data on IRF3 signaling in hepatic stellate cells has been published yet.

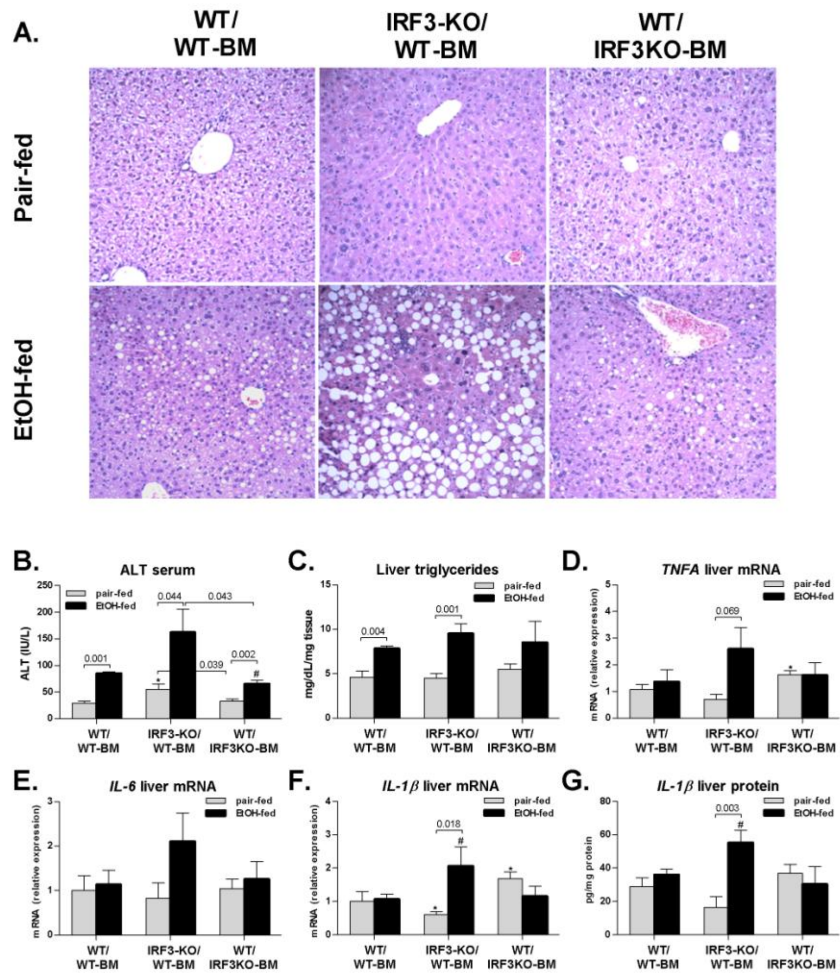


Fig. 3. Incomplete protection from alcohol-induced liver injury in mice with selective deficiency of IRF3 in bone-marrow derived cells. Figure represents an extension of Figure 2 from Enclosure 2. Wild-type mice with transplanted WT bone marrow (WT/WT-BM), *Irf3*-deficient mice with transplanted wild-type bone marrow (IRF3-KO/WT-BM) and WT mice with transplanted IRF3-KO bone marrow (WT/IRF3KO-BM) were fed Lieber DeCarli ethanol or control (pair-fed) diet and sacrificed after 4 weeks. Livers were stained with H&E; magnification 200x (A). Serum ALT levels (B) and liver triglycerides (C) were analyzed. Messenger RNA levels of liver (D) tumor necrosis factor α (TNFA), (E) interleukin 6 (IL-6) and (F) interleukin-1β (IL-1β) were analyzed by real-time PCR and normalized to 18s. Liver IL-1β levels were analyzed using ELISA (G). Values are shown as mean ± SEM fold increase over wild-type pair-fed control group (5-7 mice per group). Numbers in graphs denote p values; *) $p < 0.05$ vs. pair-fed wild-type mice; #) $p < 0.05$ vs. ethanol-fed wild-type mice.

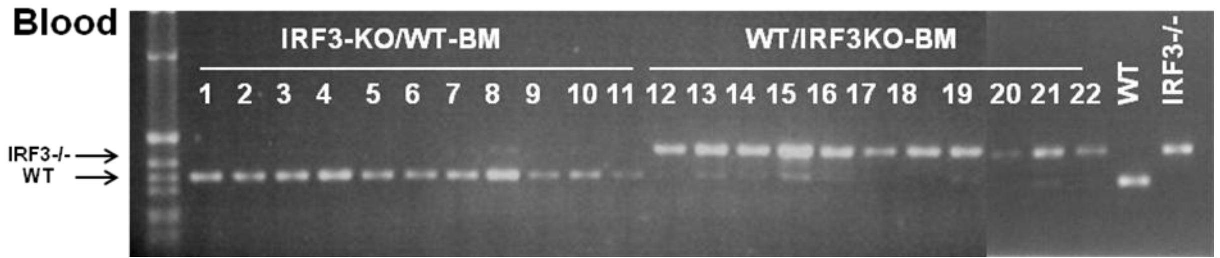


Fig. 4. Engraftment of transplanted bone-marrow was confirmed from DNA isolated from peripheral blood using primers specific for wild-type and truncated form of IRF3. Samples 20, 21, 22 together with the WT and IRF3-/- control samples were run in a different row of the same agarose gel and were collated to the rest of the samples to enable comparison.

4.2.3 Conclusion

In conclusion, our results demonstrate a cell-specific role of IRF3 in the pathogenesis of alcoholic-induced liver injury. Global *Irf3* deficiency confers protection from liver injury induced by alcohol, most likely as a result of reduced induction of pro-inflammatory cytokines. On the other hand, hepatocyte-specific IRF3 activation and type I IFN induction have protective effects in ALD. Disruption of IRF3 in hepatocytes decreases type I IFN induction and increases liver injury due to dysregulated expression of pro- and anti-inflammatory cytokines (24).

The results therefore confirm the hypothesis IRF3 is indispensable for the pathogenesis of ALD, and suggest that IRF3 is the key transcription factor mediating the pro-inflammatory effect of the TLR4- /TRIF-dependent signaling. In addition, the study emphasizes the active role of hepatocytes in modulating the extent of the innate immune response elicited by LPS/TLR4.

4.3. Specific aim C - The role of probiotics in modulation of fibrosis in non-alcoholic steatohepatitis

Enclosure 3: Velayudham A, Dolganiuc A, Ellis M, **Petrasek J**, Kodys K, Mandrekar P, et al. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. Hepatology 2009;49:989-997.

Non-alcoholic fatty liver disease and NASH are frequent causes of chronic liver diseases in Western world. NASH features the metabolic syndrome and liver inflammation. Individuals with advanced stages of NASH develop fibrosis, which may progress to cirrhosis and to end-stage liver disease (247). Given the fact that gut-derived bacterial components contribute to the pathogenesis of NASH, modulation of intestinal microbiota could represent a potential therapeutic approach in NASH.

4.3.1. Summary of results

We tested the hypothesis that probiotic VSL#3 may ameliorate the MCD diet–induced mouse model of NASH. MCD diet resulted in NASH in WT mice compared to MCS diet feeding. This was evidenced by liver steatosis, increased triglycerides, inflammatory cell accumulation, increased TNF-α levels, and fibrosis. The VSL#3 probiotic diet failed to prevent MCD-induced liver steatosis or inflammation. In contrast, VSL#3 treatment ameliorated MCD diet–induced liver fibrosis resulting in diminished accumulation of collagen and α-smooth muscle actin. Thus, VSL#3 treatment prevented fibrosis in the MCD diet-induced NASH without significant attenuation of the ongoing steatohepatitis. This observation supports the concept that *in vivo* fibrosis and steatohepatitis can be regulated independently (310), and points to a potentially new therapeutic application of VSL#3.

4.3.2. Discussion

The current concept of the mechanism of NASH favors a model in which steatosis and steatohepatitis are induced as a result of fatty acid overload and inflammation, leading to subsequent activation of HSCs that produce extracellular matrix and lead to liver fibrosis

(22). Activation of HSCs is induced by multiple insults, including TNF-α and transforming growth factor beta (TGF-β) (16). We identified increased production of TNF-α production in mice with MCD diet-induced NASH, which remained elevated in MCD + VSL#3-treated mice in agreement with previous reports (247). Also, we identified that MCD diet upregulated TGF-β, a known activator of HSCs. Because TGF-β regulates collagen production (13), increased TGF-β could contribute to the MCD-induced liver fibrosis.

VSL#3 inhibited fibrosis and, importantly, triggered the production of Bambi, a transmembrane protein highly similar to TGFβ receptors (13). In contrast to regular TGFβ receptor, the intracellular domain of Bambi lacks a serine/threonine kinase domain that is essential for transducing TGF-β signals; thus, Bambi functions as a pseudoreceptor and acts as a negative regulator of TGF-β signaling pathway. To date, the mechanisms of Bambi regulation are not fully understood. However, several authors reported that Bambi is regulated via NF-κB–dependent mechanisms (13, 311). We report increased NF-κB activity and elevated expression of Bambi in MCD diet–fed VSL#3-treated group compared to controls fed the MCD diet alone. Further, Bambi RNA changes mirror the protein levels, and Bambi expression is restricted to HSCs. Thus, in the presence of VSL#3, high levels of Bambi could prevent TGF-β-induced signals, and control the unrestricted activation of HSCs by ongoing inflammation. These data are in agreement with those of Seki et al. (13), who showed that down-regulation of Bambi mRNA and protein expression, and subsequent sensitization to TGF-β signals, is mediated by a MyD88/NF-κB–dependent pathway and occurs with ongoing liver inflammation.

4.3.3. Conclusion

In summary, our data indicate that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. The mechanisms of VSL#3-mediated protection from MCD diet–induced liver fibrosis likely include modulation of collagen expression and impaired TGF-β signaling (312). Our results suggest that the beneficial effect of the VSL#3 probiotic treatment on fibrosis in the NASH model may occur even in the absence of significant changes in markers of inflammation and fat in the liver.

4.4. Specific aim D – The protective role of Type I interferons in TLR9-associated liver injury

Enclosure 4: **Petrasek J**, Dolganiuc A, Csak T, Kurt-Jones E, Szabo G. Type I Interferons Protect from Toll-like Receptor 9-Associated Liver Injury and Regulate IL-1 Receptor Antagonist in Mice. Gastroenterology 2010, doi. 10.1053/j.gastro.2010.08.020.

TLR9-dependent liver inflammation and injury is involved in the pathogenesis of alcoholic liver disease(174), non-alcoholic steatohepatitis (249), acetaminophen-induced liver injury (11), primary biliary cirrhosis (9) and primary sclerosing cholangitis (10), as well as in pathological processes such as liver fibrosis (313), liver cirrhosis (16), ischemia-reperfusion injury (14), and liver graft rejection (15), Thus, TLR9 holds the common link between different processes which lead to liver diseases; therefore, unraveling the pathogenesis of TLR9-induced liver injury may aid in identification of novel, efficient pathogenesis-based management or cure.

4.4.1. Summary of results

We investigated the role of Type I IFNs and Type I IFN-dependent downstream mediators in regulation of TLR9-associated liver injury. We evaluated liver injury and inflammation in wild-type (WT), Irf7-deficient (IRF7-KO) or IFN α/ß receptor 1 –deficient (IFNAR1-KO) - mice in a model of TLR9-dependent injury following intraperitoneal injection of TLR9 (CpG DNA) plus TLR2 (lipoteichoic acid) ligands.

We observed that Type I IFNs were upregulated during TLR9-associated liver injury in WT mice. IRF7- and IFNAR1-deficient mice, that have disrupted Type I IFN induction and signaling, respectively, exhibited exaggerated TLR9-induced liver damage and inflammation, associated with significantly lower recruitment of dendritic cells into the liver, and increased production of TNF-α by LMNCs. These findings indicated that Type I IFNs have anti-inflammatory activities in liver.

Interleukin 1-receptor antagonist (IL-1ra), which is produced by LMNCs and hepatocytes, is an IFN-regulated antagonist of the pro-inflammatory cytokine IL-1β. We identified decreased IL-1ra in IRF7- and IFNAR1-deficient mice downstream of TLR9, compared to WT mice, suggesting an imbalance in IL-1β/IL-1ra signaling and preferential

proinflammatory activation. IL-1ra protected cultured hepatocytes from IL-1β-mediated sensitization to cytotoxicity from TNF-α. *In vivo* administration of Type I IFN to WT mice induced IL-1ra and significantly ameliorated TLR9-associated liver injury. The protective role of IL-1ra was confirmed *in vivo* where administration of recombinant IL-1ra protected against TLR9-associated liver injury suggesting that the anti-inflammatory effect of Type I IFNs could be mediated by IFN-dependent induction of IL-1ra.

4.4.2. Discussion

An important role for TLR9 is recognized in a variety of liver disease and this makes the current novel finding (Enclosure 4) of significance. In our model of TLR9-induced liver injury, we have convincingly shown an IRF7-mediated increase of Type I IFNs resulting in a significant reduction in liver injury by upregulation of IL-1ra. In the framework of our research projects, this is the second set of experiments that provides evidence for anti-inflammatory role of Type I IFNs in the liver. In addition, the present study presents novel concepts: First, both liver mononuclear cells and hepatocytes are the cellular sources of IL-1ra in the liver. Second, Type I IFNs are involved in sensitization of hepatocytes to cell death induced in the context of TLR9-associated liver injury, and IL-1ra has a protective effect in this process. Third, recruited inflammatory cells are sensitized to induce tumor necrosis factor α after TLR9 priming in IRF7- and IFNAR1-dependent manner. Last, dendritic cells are preferentially recruited to TLR9-induced liver in a CCL21/CCR7-dependent manner.

4.4.2.1. The TLR9-associated liver injury model used in our research project

Although the current model is being presented as TLR9-associated liver injury, three TLR agonists are being used (TLR9, TLR2 and TLR4). Our recent and previous (173, 257) data show that liver inflammation and injury induced in this model are primarily dependent on TLR9; liver damage elicited by CpG is aggravated by co-stimulation with TLR2 and a secondary stimulation with TLR4. Of these TLRs, TLR2, 4 and 9 activate the MyD88-dependent pathway, while IRF3 activation is exclusive to TLR4. Type I IFN and IRF7 induction can occur via both TLR9 and TLR4 (314).

4.4.2.2. The contribution of IRF7 and IFNAR1 to the sensitization of hepatocytes to cell death

Our findings of increased liver inflammation and damage suggested a protective role of IRF7 and IFNAR1 in cell death of hepatocytes. To address this question, we isolated hepatocytes from WT, IRF7 and IFNAR1-deficient mice treated in vivo with saline or CpG+LTA (Fig. 1). *Ex vivo* culture of saline-treated WT hepatocytes on collagen-coated plates for 24 hours lead to some LDH release, suggestive of cell death. LDH release was significantly lower in IRF7- and IFNAR1-deficient hepatocytes compared to WT. These findings indicated that absence of IRF7 and IFNAR1 in hepatocytes per se does not induce spontaneous hepatocyte death. In contrast, IRF7- and IFNAR1-deficient hepatocytes isolated from CpG+LTA-primed mice showed a significantly higher LDH release, compared to WT hepatocytes (Fig. 1). These findings indicated that absence of IRF7 and IFNAR1 in hepatocytes is involved in sensitization of hepatocytes to cell death induced in the context of TLR9-associated liver injury. These data provided us with the background to explore the mechanistic aspects of the phenomenon.

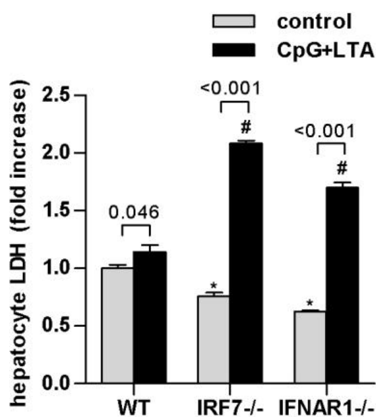


Fig. 1. WT, IRF7- and IFNAR1-deficient mice were primed with saline or CpG+LTA i.p. After 3 days, primary hepatocytes were isolated and plated on collagen-coated plates. LDH release into cell culture supernatant was measured after 24 hours and normalized to total LDH. Representative values from total n= 4 per group are shown as average ± SEM. *) P <0.05 vs WT control, #) P < 0.05 vs WT CpG+LTA.

To further dissect the mechanism, we tested the hypothesis that the increased sensitivity of hepatocytes to death is a consequence of enhanced pro-inflammatory signals in IRF7- and IFNAR1-deficient mice and/or of insufficient anti-inflammatory signals. This hypothesis derived from our observation of significantly enhanced *ex vivo* induction of TNF-α in liver mononuclear cells isolated from IRF7- and IFNAR1-deficient mice, compared to WT mice (Fig. 6B in Enclosure 4), thus we further asked whether TNF-α per se could contribute to hepatocyte death. Consistent with previous reports that healthy hepatocytes are

resistant to stimulation with TNF-α alone (215), we observed that increasing doses of recombinant TNF-α did not induce death in WT hepatocytes (Fig. 6D in Enclosure 4).We also noted that IL-1β, which has been induced in our model of TLR9-associated liver injury (Fig. 5A,B in Enclosure 4), could sensitize hepatocytes to cell death induced by TNF-α (Fig. 6D,E in Enclosure 4). We further report that pretreatment with recombinant IL-1ra significantly decreased WT hepatocyte death induced by IL-1β and TNF-α (Fig. 6D,E in Enclosure 4). Based on these data, we conclude that the protective role of IRF7 and IFNAR1 in hepatocyte death in TLR9-associated liver injury is mediated by Type I IFN-dependent induction of IL-1ra, which opposes the IL-1β –dependent sensitization of hepatocytes to TNF-α –induced cell death.

4.4.2.3. Cell types recruited into the TLR9-associated liver inflammatory infiltrates

The increased inflammatory infiltration in IRF7- and IFNAR1-deficient mice stimulated with TLR9 + TLR2 ligands, compared to WT animals, lead us to investigate the types of recruited inflammatory cells. We employed flow cytometry analysis and observed that both WT and IFNAR1-KO mice showed comparable proportion/activation of monocytes/macrophages, as indicated by the expression of CD68 (Fig. 4A in Enclosure 4). This finding was compatible with the lack of significant differences in expression of chemokines and chemokine receptors (Supplementary Fig. 1 in Enclosure 4) involved in trafficking of monocytes/ macrophages. In contrast, we observed a significantly decreased recruitment of myeloid and plasmacytoid dendritic cells in IFNAR1-deficient animals (Fig. 4B in Enclosure 4). This finding suggested a role of Type I IFNs in recruitment of dendritic cells in the context of TLR9-associated injury.

To search for possible explanation, we analyzed liver expression of the chemokine ligand 21 (CCL-21) and its receptor CCR7, which are critically involved in trafficking of dendritic cells (315), and observed a significant downregulation of CCL-21 in the livers of IFNAR1-deficient mice (Fig. 4C,D in Enclosure 4).

Our finding of decreased CCL-21 expression in the liver of mice deficient in IFNAR1 implied involvement of Type I IFNs in regulation of this chemokine. We further performed a database search (Genecards) which showed that the gene for human CCL-21 contains a Type I IFN-responsive element for IRF7 in its promoter (Fig 2). CCL-21 is a chemokine produced predominantly in lymphatic endothelium or in high endothelial venules in liver portal tracts (316). Using a murine lymphatic endothelium cell line SVEC4-10, we demonstrated that

IFN-α significantly induced CCL-21 (Fig. 3). Our data suggest a novel Type I IFN-dependent mechanism for dendritic cell recruitment into the liver mediated by CCL-21.

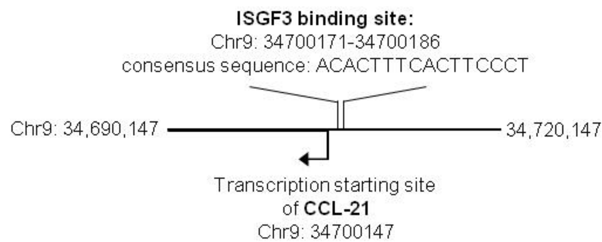


Fig. 2. Position of the ISGF-3 binding site in human CCL21 gene promoter. Adapted from Genecards (www.genecards.org), accession number GC09M034699. Accessed 7/2010.

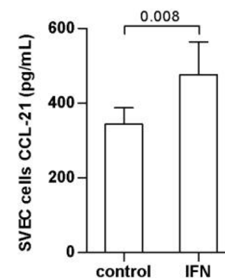


Fig. 3. Murine SVEC4-10 cells were stimulated with mouse IFN-α2b for 6 hours. Protein levels of CCL-21 were measured using ELISA. Values are shown as mean ± SEM.

4.4.2.4. Mechanisms of sensitization of TLR9+TLR2 ligands to LPS

Our results have shown that administration of LPS to mice previously primed with TLR9 + TLR2 ligands significantly aggravated liver injury. This finding led us to the hypothesis that recruited inflammatory cells are sensitized after TLR9+TLR2 treatment. We observed that LMNCs isolated from mice pretreated with TLR9+TLR2 ligands in vivo and stimulated with LPS *ex vivo* showed a significant induction of TNF-α and IL-1β, compared to cells from non-sensitized mice *ex vivo* stimulated with LPS, or in comparison to cells from sensitized mice without *ex vivo* stimulation (Fig. 6B,C in Enclosure 4). We observed significantly higher production of TNF-α after ex vivo stimulation with LPS, in cells isolated from CpG+LTA-primed mice deficient in IRF7 or in IFNAR1 compared to cells isolated from WT mice (Fig. 6B in Enclosure 4). Production of IL-1β (Fig. 6C in Enclosure 4) did not differ between genotypes, which is in line with distinct regulatory mechanisms involved in regulation of TNF-α vs. IL-1β (317).

4.4.2.5. The role of dendritic cells in liver damage/inflammation/fibrosis

In our study, we observed a significantly decreased recruitment of both conventional and plasmacytoid dendritic cells into the liver of IFNAR1-deficient mice with TLR9-associated liver injury, compared to wild-type mice. This finding suggested that dendritic cells may have a protective role in the liver. However, Yoneyama et al. (316) demonstrated that dendritic cell infiltration is essential for formation of inflammatory infiltrate and inducing Th1 type inflammatory response in *Propionibacterium acnes* treated livers (CpG +

LTA model mimics this model). In addition, Connolly et al. (140) demonstrated that depletion of dendritic cells reduced liver fibrosis, indicating that dendritic cells induce inflammation. Therefore it is unclear whether dendritic cells are required for pro-inflammatory or anti-inflammatory signaling in the liver.

Consistent with earlier observation (173), TLR9-induced inflammatory infiltrates that require dendritic cell recruitment to the liver (318), amplified pro-inflammatory cytokine induction by LPS. Our data therefore support the previously reported pro-inflammatory role of dendritic cells (316). On the other hand, our novel data indicate a yet unrecognized dual role for dendritic cells by providing anti-inflammatory signals in the liver. Our data also imply a potential role of CCL-21 and CCR7 in liver injury. To the best of our knowledge, the role of inactivation of this chemokine/chemokine receptor pair is currently unknown.

4.4.3. Conclusion

In summary, our findings imply that the endogenous anti-inflammatory signaling induced by Type I IFNs and mediated by IL-1ra regulates the extent of TLR9-induced liver damage, and support the indispensable role of Type I interferon signaling in immune-mediated liver injury. Our data also suggest a potential role for IL-1ra in therapy of TLR9-associated liver diseases.

5. Conclusions

Within the subject of Toll-like receptors in the pathogenesis of liver injury, our work resulted in and contributed to the following points:

A.We determined that hereditary susceptibility caused by common variants in genes involved in TLR4-induced pathogenesis of alcoholic liver cirrhosis is low in central European population.

a. Small or pilot allelic association studies frequently result in positive findings that are not replicable in larger cohorts.
b. Performing allelic association studies in meta-analyses or multicentric studies increases the probability of detecting a true positive association; however, statistically significant results from such studies may be of low biological significance in the context of stronger demographic and clinical determinants.

B. We showed that hepatocyte-derived Type I interferons play anti-inflammatory role in the pathogenesis of alcohol-induced liver disease in mice.

a. The pathogenesis of alcohol-induced liver disease is critically dependent on the interferon regulatory factor 3 (IRF3), an alternative downstream mediator of TLR4 activation.
b. IRF3 in bone-marrow derived cells in the liver has pro-inflammatory role in alcohol-induced liver disease.
c. Hepatocyte-specific IRF3 is protective in alcohol-induced liver disease by means of induction of Type I IFNs that induce the anti-inflammatory IL-10 in mononuclear cells, thus diminishing the extent of alcohol-induced liver inflammation and injury.
d. In the context of alcohol-induced liver disease, the crosstalk between hepatocytes and bone-marrow derived cells is critical in regulating the extent of liver inflammation and damage induced by ethanol.

<u>C. We demonstrated a differential effect of probiotic diet on inflammation and liver fibrosis in non-alcoholic steatohepatitis (NASH) model in mice.</u>

a. VSL#3 probiotic diet did not show any effect on TLR4-dependent inflammatory signaling, inflammation and steatosis in NASH.
b. VSL#3 probiotic diet significantly ameliorated NASH-associated liver fibrosis. This effect likely involved modulation of collagen expression and impaired TGF-β signaling.
c. Our results suggest that, at least in the NASH model, the benefit of the VSL#3 treatment on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver.

<u>D. We identified a protective role of Type I IFNs in immune-mediated liver injury induced by TLR9 signaling.</u>

a. Type I IFNs are significantly induced in TLR9-associated liver diseases
b. Absence of Type I IFN induction or signaling substantially aggravated TLR9-associated liver inflammation and injury, increased production of inflammatory cytokines by liver mononuclear cells, and decreased the expression of interleukin 1-receptor antagonist, which is a Type I IFN-dependent anti-inflammatory cytokine.
c. Our results demonstrated a critical anti-inflammatory and protective role of Type I IFNs and Interleukin-1 receptor antagonist in immune-mediated liver injury and suggest potential therapeutic implications.

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7. List of publications, awards and presentations

Summary

Total number of publications	23
International journals with impact factor:	14
Book chapters:	1
Total sum of impact factor points:	70.1
Papers with first authorship:	22.7
Total number of citations:	76
Average number of citations per published article:	7.6
Hirsch index:	6
Number of congress presentations:	36
International meetings:	27
Oral presentations:	14
Presentations of distinction:	12
First authorship:	26
Number of author's awards:	15
International awards:	12

a. List of author’s publications related to the topic of the Ph.D. thesis

i. Original articles

1. **Petrasek J**, Hubacek JA, Stickel F, Sperl J, Berg T, Ruf E, et al. Do common genetic variants in endotoxin signaling pathway contribute to predisposition to alcoholic liver cirrhosis? *Clin Chem Lab Med* 2009;47:398-404. **IF: 1.89**
2. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, et al. Hepatocyte-specific IRF3 and Type I interferons are protective in alcohol-induced liver injury in mice via cross-talk with macrophages. 2010; Manuscript submitted.
3. Velayudham A, Dolganiuc A, Ellis M, **Petrasek J**, Kodys K, Mandrekar P, et al. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 2009;49:989-997. **IF: 11.56**
4. **Petrasek J**, Dolganiuc A, Csak T, Kurt-Jones E, Szabo G. Type I Interferons protect from Toll-like receptor 9-associated liver injury and regulate IL-1 receptor antagonist in mice. *Gastroenterology* 2010; *doi. 10.1053/j.gastro.2010.08.020.* **IF: 12.89**

ii. Review articles

5. **Petrasek J**, Mandrekar P, Szabo G. Toll-like receptors in the pathogenesis of alcoholic liver disease. Gastroenterology research and practice 2010. doi:10.1155/2010/710381.
6. **Petrasek J.** Genetické faktory v patogenezi alkoholické nemoci jater. In: Špičák, J et al. Novinky v gastroenterologii a hepatologii. Grada publishing 2006, pp. 87-118. ISBN: 978-80-247-1783-8

b. List of author’s publications not related to the topic of the Ph.D. thesis

1. Dolganiuc A, **Petrasek J**, Kodys K, Catalano D, Mandrekar P, Velayudham A, et al. MicroRNA expression profile in Lieber-DeCarli diet-induced alcoholic and

methionine choline deficient diet-induced nonalcoholic steatohepatitis models in mice. Alcohol Clin Exp Res 2009;33:1704-1710. **IF: 3.33**

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5. Sperl J, **Petrasek J**, Spicak J, Viklicky O. Acute rejection of non-functional allograft in kidney transplant recipients with hepatitis C treated with peginterferon-alpha 2a. J Hepatol 2008;49:461-462; author reply 462-463. **IF: 6.44**
6. Dezortova M, Jiru F, **Petrasek J**, Malinova V, Zeman J, Jirsa M, et al. 1H MR spectroscopy as a diagnostic tool for cerebral creatine deficiency. Magma 2008;21:327-332. **IF: 1.84**
7. Vitek L, Novotna M, Lenicek M, Novotny L, Eberova J, **Petrasek J**, et al. Serum bilirubin levels and UGT1A1 promoter variations in patients with schizophrenia. Psychiatry Res 2010; in press. **IF: 2.66**
8. **Petrasek J**, Hucl T, Spicak J. Pancreaticobiliary malunion and incomplete pancreas divisum: an unusual cause of common bile duct obstruction. Adv Med Sci 2008;53:6-10.
9. Viklicky O, Hribova P, Volk HD, Slatinska J, **Petrasek J**, Bandur S, et al. Molecular phenotypes of acute rejection predict kidney graft prognosis. J Am Soc Nephrol 2010;21:173-180. **IF: 7.51**
10. Bandur S*, **Petrasek J***, Hribova P, Novotna E, Brabcova I, Viklicky O. Haplotypic structure of ABCB1/MDR1 gene modifies the risk of the acute allograft rejection in renal transplant recipients. Transplantation 2008;86:1206-1213. **IF: 3.73 *these authors contributed equally**
11. Hribova P, Reinke P, **Petrasek J**, Brabcova I, Hubacek JA, Viklicky O. Heme oxygenase-1 polymorphisms and renal transplantation outcomes: balancing at the detection limit of allelic association studies. Am J Transplant 2008;8:1077-1078; author reply 1079. **IF: 6.28**

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13. Kopeckova M, Paclt I, **Petrasek J**, Pacltova D, Malikova M, Zagatova V. Some ADHD polymorphisms (in genes DAT1, DRD2, DRD3, DBH, 5-HTT) in case-control study of 100 subjects 6-10 age. Neuro Endocrinol Lett 2008;29:246-251. **IF: 1.25**
14. **Petrasek J**, et al. Role of genetic factors in the pathogenesis of alcoholic liver disease.World Gastroenterology News, 2004, vol. 9, no. 2, p. 11-12.
15. **Petrasek J**, et al. Nejčastější dědičné choroby jater a trávicího traktu. Bulletin Sdružení praktických lékařů ČR, 2006, vol. 16, no. 2, p. 50-55.
16. **Petrasek J**, et al. Molekulární diagnostika nejčastějších dědičných chorob trávicí soustavy: I. Choroby jater. Praktický lékař, 2006, vol. 86, no. 10, p. 588-590.
17. **Petrasek J**, et al. Molekulární diagnostika nejčastějších dědičných chorob trávicí soustavy: II. Kolorektální karciniom a choroby pankreatu. Praktický lékař, 2006, vol. 86, no. 11, p. 654-656.
18. **Petrasek J**, et al. Polymorfismy v genovém clusteru pro interleukin 1: rizikový faktor alkoholické jaterní cirhózy. Čes. a Slov. Gastroent. a Hepatol. 2007, vol. 61, no. 2, p. 26 – 31.

c. List of author's awards

2010 The Research Society on Alcoholism award. 33rd Annual RSA Scientific Meeting, San Atonio, Texas, U.S.A. Oral presentation.

2010 Cena Jana Opletala (Jan Opletal Award), 1st Faculty of Medicine, Charles University Prague, Czech republic. Distinguished Ph.D. student award.

2010 American Gastroenterological Association award (oral presentation). Digestive Disease Week (DDW).

2010 European Association For The Study Of The Liver young investigator travel award. Monothematic conference: Signaling in the Liver

2009 American Gastroenterological Association award (oral presentation). Digestive Disease Week (DDW).

2009 American Association for the Study of Liver Diseases award (poster of distinction).

AASLD Liver meeting.

2008 European Association For The Study Of The Liver young investigator travel award. EASL liver week.

2008 American Gastroenterological Association award (poster of distinction). Digestive Disease Week (DDW).

2008 Czech hepatology society award (best original article published in an international journal in 2007)

2008 1. cena Nadačního fondu Scientia (Scientia foundation Distinguished Ph.D. student award). 1st Faculty of Medicine, Charles University Prague.

2007 Slovak gastroenterological society for the best poster presentation. Gastroforum meeting 2007

2007 European Association For The Study Of The Liver young investigator travel award. EASL liver week.

2007 American Gastroenterological Association award (poster of distinction). Digestive Disease Week (DDW).

2006 European Association For The Study Of The Liver young investigator travel award. EASL liver week.

2006 Falk foundation award for the 2nd best poster presentation. Falk Symposiun No. 156: genetics in Liver Diseases.

2006 8th European Bridging Meeting in Gastroenterology Award for the best oral presentation.

d. List of author's presentations

1. **Petrasek J**, Csak T, Kurt-Jones, E, Szabo, G. Interferon regulatory factor 3 signaling promotes liver fibrosis in mice and regulates the chemokine ligand 21 via Type-I interferons. The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), November 2010, Boston, Massachusetts, U.S.A. **Poster presentation.**
2. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, Mandrekar P, Szabo G. Hepatocyte-specific IRF3 signaling protects from alcohol-induced liver injury in mice through regulation of monocyte/macrophage-derived cytokines via Type-I

interferons. The 33rd Annual RSA Scientific Meeting, 28.6.2010, San Atonio, Texas, U.S.A. **Oral presentation**.

3. **Petrasek J**, Dolganiuc A, Kurt-Jones, E, Szabo, G. Type I Interferons Regulate IL-1 Receptor Antagonist and Protect from Toll-like Receptor 9-Mediated Liver Injury in Mice. *Digestive Disease Week 2010 (DDW)*, 2.5.2010, New Orleans, U.S.A. **Oral presentation.**
4. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, Mandrekar P, Szabo G. Protective effect of hepatocyte-specific Type-I interferon in alcohol-mediated liver injury. The Henry and Lillian Stratton basic research conference of the American Association for the Study of Liver Disease (AASLD), 12.9.2009 – 14.9.2009, Atlanta, Georgia, U.S.A. **Poster.**
5. **Petrasek J**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, Mandrekar P, Szabo G. Hepatocyte-specific IRF3 signaling protects from alcohol-induced liver injury in mice through regulation of monocyte/macrophage-derived cytokines via Type-I interferons. The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Poster of distinction.**
6. **Petrasek J**, Dolganiuc A, Kurt-Jones, E, Szabo, G. Type I Interferon Induction and Signaling Protect from Liver Injury and Granulomas Through Regulation of IL-1 Receptor Antagonist and Dendritic Cell Recruitment in Mice. Digestive Disease Week 2009 (DDW), 2.6.2009, Chicago, U.S.A. **Oral presentation.**
7. Csak T, Dolganiuc A, Nath B, **Petrasek J**, Mandrekar P, Szabo G. Non alcoholic steatohepatitis sensitizes to poly I:C induced liver damage and leads to decreased antiviral immune response by reducing mitochondrial association of the antiviral signaling protein (MAVS). The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Poster of distinction.**
8. Nath B, Levin I, Csak T, **Petrasek J**, et al. Hypoxia inducible factor-1α is a determinant of lipid accumulation and liver injury in alcoholic steatosis. The Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Oral presentation.**
9. Marcos M, Bala S, Kodys K, Catalano D, **Petrasek J, et al** . MicroRNA-155 in Kupffer cells mediates increased TNF-alpha production in alcoholic liver disease. *The*

Liver Meeting 2009, American Association for the Study of Liver Disease (AASLD), 30.10.2009 - 3.11.2009, Boston, Massachusetts, U.S.A. **Poster.**

10. **Petrasek J**, et al. Temporary nasobiliary drainage induces relief from severe long-lasting pruritus in drug-induced canalicular cholestasis. *43rd Annual Meeting of the European Association For The Study Of The Liver (EASL)*, Milano, Itálie, 23. - 27. 4. 2008. **Poster.**
11. **Petrasek J**, et al. N-acetyl cysteine ameliorates liver injury in patients with erythropoietic protoporhyria. *Digestive Disease Week 2008 (DDW)*, 17. - 22. 5. 2008, San Diego, U.S.A. **Poster.**
12. **Petrasek J**, et al. Polymorfismy v genovém clusteru interleukinu 1: rizikový faktor alkoholické jaterní cirhózy. *XII. Gastrofórum 2007,* Štrbské pleso, SR, 23. – 26. 1. 2007. **Poster.**
13. **Petrasek J**, et al. Hypersekretorní haplotyp genového clusteru interleukinu 1: rizikový faktor alkoholické cirhózy jater. *XI. Hradecké gastroenterologické dny*, Hradec Králové, 16. -17. 3. 2007. **Oral presentation.**
14. **Petrasek J**, et al. Hypersecretory haplotype of the IL-1 gene cluster increases the risk of alcoholic liver cirrhosis. *Digestive Disease Week 200*7, 19. - 24. 5. 2007, Washington, U.S.A. **Poster.**
15. **Petrasek J**, et al. High secretory haplotype of interleukin-1 gene cluster increases the risk of alcoholic liver disease. *42nd Annual Meeting of the European Association For The Study Of The Liver*, Barcelona, Spain, 11. - 15. 4. 2007. **Poster.**
16. **Petrasek J**, et al. Hypersekretorní haplotyp genového clusteru interleukinu-1: rizikový faktor alkoholické cirhózy jater. XXXV. Májové hepatologické dny, 9.-12.5.2007, Karlovy Vary. **Oral presentation.**
17. **Petrasek J**, et al. Hypersecretory haplotype of interleukin-1 gene cluster increases the risk of alcoholic liver cirrhosis. *11th Congress of the European Society for Biomedical Research on Alcoholism (ESBRA),* Berlín, Německo, 23. – 26. 9.2007. **Oral presentation.**
18. Paclt I, Kopeckova M, **Petrasek J**, et al. ADHD polymorhpisms in case control study of 100 subjects 6-10 age. *Celostátní sjezd Společnosti lékařské genetiky ČLS JEP,* Praha, 19. – 21.9.2007. **Poster.**
19. **Petrasek J**, et al. Revised King´s college score for liver transplantation in adult patients with Wilson´s disease. *13th Congress of the European Society for Organ Transplantation.* Prague, 29.9. – 3.10.2007. **Oral presentation.**

20. Brabcova I, **Petrasek J**, et al. Cytokine and chemokine gene variants and kidney graft outcome. *13th Congress of the European Society for Organ Transplantation (ESOT),* Prague, 29.9 – 3.10.2007. **Poster.**
21. Sperl J, Frankova S, **Petrasek J**, et al. Liver transplantation for epitheloid hemangioendothelioma: a single-centre experience. *13th Congress of the European Society for Organ Transplantation (ESOT),* Praha, 29.9 – 3.10.2007. **Poster.**
22. **Petrasek J**, et al. Nucleoside analogues in acute hepatitis B. *9th European Bridging Meeting in Gastroenterology.* 22.11-24.11.2007, Magdeburg, Germany. **Oral presentation.**
23. **Petrasek J**, et al. Lamivudine has no effect on the course and outcome of acute fulminant hepatitis B. *9th European Bridging Meeting in Gastroenterology.* 22.11-24.11.2007, Magdeburg, Germany. **Poster**.
24. Hejlova I, **Petrasek J**, et al. Budd-Chiari sydnrome as an indication for liver transplantation – single center experience. *9th European Bridging Meeting in Gastroenterology.* 22.11-24.11.2007, Magdeburg, Germany. **Poster**.
25. **Petrasek J**, et al. Úloha polymorfismů modulujících odpověď na stimulaci endotoxiny v patogenezi alkoholické nemoci jater. *XXXIV. Májové hepatologické dny*, Karlovy Vary, 3. – 5. 5. 2006. **Oral presentation.**
26. **Petrasek J**, et al. Zhodnocení diagnostických a prediktivních indexů u pacientů s fulminantní a dekompenzovanou chronickou Wilsonovou chorobou. *XXXIV. Májové hepatologické dny*, Karlovy Vary, 3. – 5.5.2006. **Oral presentation.**
27. **Petrasek J**, et al. Diagnostic and predictive indexes in patients with fulminant and decompensated Wilson´s disease. *41st Annual Meeting of the European Association For The Study Of The Liver,* Vídeň, Rakousko, 26-30.4.2006. **Poster.**
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31. **<u>Petrasek J</u>**, et al. Je fibróza parenchymatózních orgánů potenciálně reverzibilní? *9. vzdělávací a diskusní gastroenterologické dny*, Karlovy Vary, 16.-18. 11. 2006. **Oral presentation.**
32. **<u>Petrasek J</u>**, et al. High secretory haplotype of interleukin-1 gene cluster increases the risk of alcoholic liver cirrhosis. *8th European Bridging Meeting in Gastroenterology*, Neapol, Itálie, 23. - 24. 11. 2006. **Oral presentation.**
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34. **<u>Petrasek J</u>**, et al. The role of genetic factors in pathogenesis of the alcoholic liver disease. 32. Májové hepatologické dny, Karlovy Vary, 2. - 14. 5. 2004. **Oral presentation.**
35. **<u>Petrasek J</u>**, et al. Association of uncoupling protein-2 and superoxide dismutase-2 polymorphisms with susceptibility to alcoholic liver disease. *12th United European Gastroenterology Week*, Prague, 25. - 29. 9. 2004. **Poster.**

8. Enclosures

1. **<u>Petrasek J</u>**, Hubacek JA, Stickel F, Sperl J, Berg T, Ruf E, et al. Do common genetic variants in endotoxin signaling pathway contribute to predisposition to alcoholic liver cirrhosis? *Clin Chem Lab Med* 2009;47:398-404. **IF: 1.89**
2. **<u>Petrasek J</u>**, Dolganiuc A, Nath B, Hritz I, Kodys K, Catalano D, et al. Hepatocyte-specific IRF3 and Type I interferons are protective in alcohol-induced liver injury in mice via cross-talk with macrophages. 2010; Manuscript submitted.
3. Velayudham A, Dolganiuc A, Ellis M, **<u>Petrasek J</u>**, Kodys K, Mandrekar P, et al. VSL#3 probiotic treatment attenuates fibrosis without changes in steatohepatitis in a diet-induced nonalcoholic steatohepatitis model in mice. *Hepatology* 2009;49:989-997. **IF: 11.56**
4. **<u>Petrasek J</u>**, Dolganiuc A, Csak T, Kurt-Jones E, Szabo G. Type I Interferons protect from Toll-like receptor 9-associated liver injury and regulate IL-1 receptor antagonist in mice. *Gastroenterology* 2010, doi. 10.1053/j.gastro.2010.08.020. **IF: 12.89**

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Do common genetic variants in endotoxin signaling pathway contribute to predisposition to alcoholic liver cirrhosis?

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Abstract

Background: Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), produced by endotoxin-activated Kupffer cells, play a key role in the pathogenesis of alcoholic liver cirrhosis (ALC). Alleles *TNFA* –238A, *IL1B* –31T and variant *IL1RN**2 of repeat polymorphism in the gene encoding the IL-1 receptor antagonist increase production of TNF-α and IL-1β, respectively. Alleles *CD14* –159T, *TLR4* c.896G and *TLR4* c.1196T modify activation of Kupffer cells by endotoxin. We confirmed the published associations between these common variants and genetic predisposition to ALC by means of a large case-control association study conducted on two Central European populations.

Methods: The study population comprised a Czech sample of 198 ALC patients and 370 controls (MONICA project), and a German sample of 173 ALC patients and 331 controls (KORA-Augsburg), and 109 heavy drinkers without liver disease.

Results: Single locus analysis revealed no significant difference between patients and controls in all tested loci. Diplotype [*IL1RN**2/*2; *IL1B* –31T+] was associated with increased risk of ALC in the pilot study, but not in the validation samples.

Conclusions: Although cytokine mediated immune reactions play a role in the pathogenesis of ALC, hereditary susceptibility caused by variants in the corresponding genes is low in Central European populations.

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Keywords: alcoholic; genetic; interleukin-1β; liver cirrhosis; polymorphism; tumor necrosis factor-α.

Introduction

Recent studies on epidemiology of alcoholic liver disease suggest that only 40% of heavy drinkers will develop steatohepatitis and less than 5% will develop cirrhosis (1, 2). In addition to the cumulative dose of alcohol, gender and certain comorbidities including viral hepatitis and obesity, and yet unidentified genetic factors account for at least 50% of the individual susceptibility to alcoholic liver cirrhosis (ALC) (3, 4). Numerous studies have focused on genetic variability of the genes encoding ethanol metabolizing enzymes, genes affecting the severity of liver steatosis, oxidative stress and fibrosis, as well as genes modifying the response to endotoxins (lipopolysaccharide, LPS) (3, 5). However, the results of candidate gene case-control studies have been either inconclusive or non-replicable (6, 7).

The importance of endotoxins and activation of Kupffer cells in the pathogenesis of ALC is evidenced by experimental studies in animals (8–10) and humans (10, 11). Adachi et al. (12, 13) showed that Kupffer cell inactivation by gadolinium chloride, intestinal sterilization and targeted disruption of the genes encoding the LPS recognition receptor CD14 and the toll-like receptor 4 (TLR4) protected the animals from alcohol induced liver injury (14, 15). Similarly, mice knocked-out for tumor necrosis factor-α (TnfA) (16), type I-TnfA receptor (17), or interleukin-1β (IL-1B) (18) were resistant to alcohol-induced liver damage, whereas interleukin-1 receptor antagonist (IL1-ra) knockout mice were more susceptible to alcohol-derived insults than their wildtype littermates (19). These data strongly support the crucial role of LPS receptors and the main inflammatory cytokines produced by Kupffer cells in mouse models of alcohol induced injury.

Several functionally relevant genetic variants have been identified in the genes for CD14, TLR4, tumor necrosis factor α (TNF-α), IL-1β and IL-1ra in humans. Accordingly, the –159C/T variation in the promoter of *CD14* enhances production of CD14 by monocytes (20), whereas two linked variations, c.896A/G and c.1196C/T, in the coding region of *TLR4* impede the activation of monocytes by LPS (21). The polymorphism –238G/A of the *TNFA* promoter increases the

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transcription of TNF-α (22). The *IL1* gene cluster on chromosome 2 contains *IL1B* and *IL1RN* genes, which encode IL-1β and its receptor antagonist IL-1ra, respectively (23). The variant –31T in the promoter of *IL1B*, which changes the wildtype sequence –31CATAA to –31TATAA, significantly increases IL-1 transcription (24). The *IL1RN* gene contains a penta-allelic 86-bp tandem repeat. The second most common *IL1RN**2 allele containing two repeats increases the secretion rate of IL-1β in vitro (25). The role of these variations in susceptibility to alcohol-induced liver injury has been suggested by several groups (26–30), with the highest disease risk attributed to the *IL-1* gene cluster polymorphisms in the Asian population (27, 28).

In our study, we sought to confirm the published associations between these common variations and genetic predisposition to ALC by means of a large case-control association study conducted on a Central European (CEU) population.

Materials and methods

Subjects

Primary assessment of allelic frequencies was performed in a pilot study. Positive associations were confirmed in validation samples. The study was approved by the institutional Review Boards of all participating centers. Written informed consent was obtained from all subjects and the study conformed to the declaration of Helsinki Ethical Guidelines.

Pilot study We consecutively included 100 Caucasian patients with ALC referred to the Institute for Clinical and Experimental Medicine, Prague, Czech Republic, from March 2004 to October 2005. Their daily alcohol consumption was more than 40 g in female patients and more than 60 g in male patients for more than 10 years. The Czech control group consisted of 180 healthy volunteers without self-reported history of liver disease. The controls were selected out of 653 individuals after being ranked by a questionnaire on reported alcohol consumption, who participated in the population-based MONICA (MONItoring of trends and determinants in CArdiovascular disease) project between March 1996 and November 1997 (31).

Validation study For validation purposes, 173 patients with ALC treated in the Department of Medicine, University of Erlangen, Germany, and the Department of Medicine, Charité, Humboldt University Berlin, Germany, were analyzed. These patients had been recruited between August 1995 and December 2003. Their daily alcohol consumption was the same as for the Czech subjects. The German control group consisted of 331 healthy volunteers without self-reported history of liver disease. The controls were selected out of 812 individuals drinking 20–40 g of ethanol/day who were part of a total number of 4261 participants of the S4 survey of the population-based KORA (KOoperative Gesundheitsforschung in der Region Augsburg) project between 1999 and 2001 (32). In addition, a second German control group consisting of 109 heavy drinkers without liver disease was included. These patients were admitted between January 1999 and December 2003 to the University of Erlangen, Germany for alcohol detoxification or for other health-related problems, such as infections and accident injuries.

Subjects were assigned to the groups of ALC according to clinical/laboratory evidence for the presence of cirrhosis evidenced by simultaneous presence of: a) clinical findings typical for liver cirrhosis (jaundice, spider naevi, ascites, encephalopathy), b) abnormal blood tests (abnormal coagulation tests, decreased serum albumin concentration and platelet count), c) abnormal liver ultrasound, and d) esophageal varices on upper gastrointestinal endoscopy. In total, 11 (5.6%) Czech and 22 (12.7%) German patients with ALC who did not meet these criteria underwent liver biopsy due to presence of cirrhosis. Liver biopsy was not performed in patients with advanced liver dysfunction (coagulopathy and ascites) who met the above-mentioned criteria. A total of 83 (42%) Czech and 96 (56%) German patients with ALC underwent liver transplantation and the diagnosis of liver cirrhosis was confirmed in all cases by explanted liver histology. Patients with positive serology of hepatitis B or C, high ferritin and elevated transferrin saturation, anti-nuclear or anti-mitochondrial antibodies, decreased level of α_1-antitrypsin and those with suspected liver cancer were excluded.

Heavy drinkers without liver cirrhosis had negative clinical findings indicating liver disease, normal blood tests and normal liver ultrasonography. Blood tests were performed while actively drinking and demonstrated normal results for alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, platelet count and prothrombin time. Elevation of γ-glutamyltransferase was not an exclusion factor. None of the heavy drinkers underwent upper gastrointestinal endoscopy or liver biopsy due to ethical reasons.

The demographic and alcohol exposure data on all studied groups are summarized in Table 1.

Genotyping

Genomic DNA was isolated from peripheral blood from all patients using the QIAamp DNA Blood Mini Kit (Qiagen GmbH, Hilden, Germany). For MONICA and KORA controls, isolated DNA was obtained. Single nucleotide polymorphisms were determined by polymerase chain reaction (PCR) followed by restriction fragment length (RFLP) analysis of the products. Genotyping of the *IL1RN* variable number of tandem repeats (VNTR) locus was performed by a PCR-based fragment length polymorphism method (see Table 2). Restriction endonucleases were purchased from Fermentas (Fermentas UAB, Vilnius, Lithuania). The PCR products of the *IL1RN* VNTR analysis were coded as follows: allele 1=4 repeats (size 442-bp), allele 2=2 repeats (270-bp), allele 3=5 repeats (528-bp), allele 4=3 repeats (356-bp), allele 5=6 repeats (614-bp). The rare alleles 3, 4 and 5, constituting less than 5% of the *IL1RN* allelic variants, were grouped in the statistical analysis.

Samples from cases and controls were included in each 96-sample batch analyzed. In order to minimize genotyping errors, blank control wells were left on the PCR plates and assays were wholly re-typed if the call rate was below 90%. Three operators, unaware of the status of the samples, independently performed the genotype assignment. Out of 3762 genotyping analyses performed, approximately 100 (2.7%) were duplicated due to the discrepancy between operators. After testing for Hardy-Weinberg equilibrium, allele frequencies were checked for consistency with data from the population of European ancestry [Utah Residents with Northern and Western European Ancestry (CEU)] from the HapMap database (33).

Statistical analysis

Two-sided power calculations at p=0.05 for 80% statistical power were performed using the DSTPLAN software (http://

Table 1 Demographic and alcohol exposure data on patients with ALC, general population controls without cirrhosis and heavy drinkers without cirrhosis.

	Alcoholic liver cirrhosis	Controls without cirrhosis	Heavy drinkers without cirrhosis
Czech study population (n=568)			
n	198	370	–
Male (%)	149 (75.3)	255 (69.0)	–
Age, years, median (IQR)	54 (26–72)[a]	50 (24–80)	–
Alcohol, g/day, median (range)	100 (75–143)[a]	13 (6–29)	–
German study population (n=613)			
n	173	331	109
Male (%)	117 (67.6)	222 (67.0)	84 (77.1)
Age, years, median (IQR)	50 (24–71)[b]	50 (30–69)	43 (22–70)
Alcohol, g/day, median (range)	100 (70–150)[c]	30 (20–40)	250 (185–335)

[a] $p<0.05$ vs. controls without liver cirrhosis; [b] $p<0.05$ vs. heavy drinkers without cirrhosis; [c] $p<0.05$ vs. controls without liver cirrhosis and vs. heavy drinkers without cirrhosis. IQR, interquartile range.

Table 2 Genotyped loci in the *CD14*, *TLR4*, *TNFA*, *IL1B* and *IL1RN* genes.

Variation Ref. dbSNP[a] id	Forward primer Reverse primer	T_m, °C Time, s	Restriction enzyme Fragment length, bp
CD14 –159C/T	5′-TTGGTGCCAACAGATGAGGTTCAC-3′	60°C	*Hae*III
rs2569190	5′-TTCTTTCCTACACAGCGGCACCC-3′	30 s	204, 201, 156
TLR4 c.896A/G	5′-GATTAGCATACTTAGACTACTACCTCCATG-3′	59°C	*Nco*I
rs4986790	5′-GATCAACTTCTGAAAAAGCATTCCCAC-3′	40 s	223, 26
TLR4 c.1196C/T	5′-GGTTGCTGTTCTCAAAGTGATTTTGGGAGAA-3′	55°C	*Hinf*I
rs4986791	5′-ACCTGAAGACTGGAGAGTGAGTTAAATGCT-3′	40 s	378, 29
TNFA –238 G/A	5′-GCCCCTCCCAGTTCTAGTTC-3′	62°C	*Bam*HI
rs361525	5′-CTCACACTCCCCATCCTCCCGGATC-3′	30 s	185, 26
IL1B –31 C/T	5′-CCCTTCCATGAACCAGAGAA-3′	60°C	*Alu*I
rs1143627	5′-GCTGAAGAGAATCCCAGAGC-3′	30 s	97, 87, 54
IL1B –511 T/C	5′-TGGCATTGATCTGGTTCATC-3′	60°C	*Ava*I
rs16944	5′-GCCCTCCCTGTTCTGTATTGA-3′	30 s	190, 60
IL1RN VNTR	5′-CCCCTCAGCAACACTCC-3′	64°C	–
156109[b]	5′-GGTCAGAAGGGCAGAGA-3′	30 s	442, 270, 528, 356, 614

[a] NCBI database of genetic variation, www.ncbi.nlm.nih.gov/SNP. [b] NCBI database of unified sequence tagged sites, www.ncbi.nih.gov/genome/STS. VNTR, variable number of tandem repeats; T_m, melting temperature.

linkage.rockefeller.edu/soft). The size of the pilot study was calculated as follows. When the odds ratio (OR) of a polymorphism was assumed to be 2, the required sample size was 100 cases and 180 controls for the polymorphism with frequency of 0.5 (*CD14* –159C/T). When the OR was assumed to be 4, the same sample size was sufficient to detect a true effect of a polymorphism with frequency of 0.03 (*TNFA* –238G/A). To confirm the association of the [*IL1RN**2/*2; *IL1B* –31T+] diplotype with ALC, we calculated the minimal size of the pilot and validation studies from the observed haplotype frequencies obtained by investigation of 100 Czech cases and 180 Czech controls and in the extended pilot study, respectively.

Hardy-Weinberg equilibrium of alleles at individual loci was evaluated using the program HWE (http://linkage.rockefeller.edu/soft). Haplotype frequencies for pairs of alleles were estimated using the software EH based on the expectation-maximization algorithm (http://linkage.rockefeller.edu/soft). Linkage disequilibrium (LD) coefficients $D'=D/D_{min\ or\ max}$ and r^2 were calculated using the MIDAS software (34). Age and median alcohol consumption between the groups was compared using the Mann-Whitney test. Male to female ratios were compared using the Fisher exact test.

All association analyses were performed by logistic regression analysis using SPSS software version 14.0 (SPSS Inc., Chicago, IL, USA). Where applicable, logistic regression analysis adjusted for age was performed. Due to the multiple testing, the level of significance in the pilot study was set at $p<0.01$. In the extended pilot and validation studies, where only one association was tested, the level of significance was set at $p<0.05$. All p-values were two-sided.

Results

In the control populations, the alleles at the individual loci were in Hardy-Weinberg equilibrium. The genotype frequencies in healthy controls for all polymorphisms were in concordance with the reference HapMap database (33). As the LD between the *IL1B* –511 and *IL1B* –31 loci in 100 Czech patients with ALC and 180 population controls was perfect ($D'=1$, $r^2=1$), we investigated only the *IL1B* –31 polymorphism in both the pilot and validation samples. A significant ($p<0.0001$) but weak LD ($D'<0.5$) was found between the *IL1B* –31 and *IL1RN* VNTR loci.

Single locus analysis

Single locus analysis was performed in 100 Czech patients with ALC and 180 population controls (see Table 3). The proportion of the *TLR4* c.1196T allele was higher in patients with ALC (8.5%) than in healthy

Table 3 Genotype frequencies in 100 Czech patients with ALC and 180 Czech healthy controls.

Locus	Genotype	Patients		Controls		Association with alcoholic cirrhosis			
						Unadjusted		Adjusted for age	
		n	%	n	%[a]	p	OR (95% CI)	p	OR (95% CI)
CD14 −159	C/C	21	21.0	31	17.2		1 (reference)		1 (reference)
	C/T	43	43.0	82	45.6	0.45	0.77 (0.40–1.51)	0.37	0.73 (0.37–1.45)
	T/T	36	36.0	67	37.2	0.51	0.79 (0.40–1.58)	0.49	0.78 (0.36–1.58)
TLR4 c.896	A/A	85	85.0	160	88.8		1 (reference)		1 (reference)
	A/G	14	14.0	19	10.6	0.39	1.39 (0.67–2.90)	0.49	1.30 (0.61–2.77)
	G/G	1	1.0	1	0.6	0.66	1.88 (0.12–30.5)	0.72	1.67 (0.10–27.2)
TLR4 c.1196	C/C	85	85.0	166	92.2		1 (reference)		1 (reference)
	C/T	13	13.0	13	7.2	0.11	1.95 (0.87–4.40)	0.13	1.91 (0.83–4.37)
	T/T	2	2.0	1	0.6	0.27	3.90 (0.35–43.7)	0.21	4.79 (0.41–56.6)
TNF −238	G/G	91	91.0	170	94.4		1 (reference)		1 (reference)
	G/A	9	9.0	10	5.6	0.28	1.68 (0.66–4.29)	0.28	1.17 (0.65–4.48)
	A/A	0	0.0	0	0				
IL1B −511	T/T	14	14.0	23	12.8		1 (reference)		1 (reference)
	C/T	45	45.0	82	45.6	0.83	0.92 (0.43–1.96)	0.98	1.01 (0.47–2.19)
	C/C	41	41.0	75	41.7	0.74	0.88 (0.41–1.89)	0.97	0.98 (0.45–2.16)
IL1B −31	C/C	14	14.0	23	12.8		1 (reference)		1 (reference)
	C/T	45	45.0	82	45.6	0.83	0.92 (0.43–1.96)	0.98	1.01 (0.47–2.19)
	T/T	41	41.0	75	41.7	0.74	0.88 (0.41–1.89)	0.97	0.98 (0.45–2.16)
IL1RN	1/1	42	42.0	82	45.6		1 (reference)		1 (reference)
	1/2	47	47.0	80	44.4	0.49	1.20 (0.72–2.02)	0.37	1.28 (0.75–2.16)
	2/2	10	10.0	11	6.4	0.21	1.82 (0.71–4.63)	0.16	1.99 (0.77–5.18)
	1/3,4,5	1	1.0	5	2.8	0.25	0.29 (0.03–2.40)	0.42	0.41 (0.05–3.57)
	2/3	0	0.0	2	1.1				

[a]The sum of the percentages may exceed 100% due to rounding. OR, odds ratio; CI, confidence interval.

controls (4.2%), but the association was not significant. For the other six loci, no significant differences in allele or genotype frequency between patients and controls were found (Table 3). In spite of a better goodness-of-fit of the regression model, adjustment for age did not significantly influence the results. Furthermore, genotyping of the *TNFA* –238A, *IL1B* –31T, *CD14* –159T and *TLR4* c.1196T loci in an extended sample of 198 Czech ALC patients and 370 Czech controls did not yield any significant association. Specifically, the *TLR4* c.1196T locus was not associated with ALC both in the heterozygous model [OR 1.56, 95% confidence interval (CI) 0.88, 2.78, p=0.13] and in the homozygous model (OR 3.93, 95% CI 0.35, 43.65, p=0.26).

Haplotype analysis In the pilot study of 100 Czech patients with ALC and 180 population controls, the [*IL1RN**2/*2; *IL1B* –31T+] diplotype, determined by homozygosity for the *IL1RN* allele 2 and carriage of the *IL1B* –31T allele, was associated with an increased risk of ALC when compared to other diplotypes in the *IL-1* gene cluster. In total, 9% of cases (compared to 1.1% of controls) revealed this diplotype, with an age-adjusted OR of 9.45 (95% CI 1.96, 45.7, p=0.005) for ALC (see Table 4). Although the power of the haplotype analysis was 85%, we were aware of the wide CI for the OR. Therefore, we evaluated the association of the diplotype in three subsequent steps.

First, we expanded the sample size of Czech patients and controls. Assuming an OR of 5, a required sample size of 198 cases and 370 controls was used. In total, 98 consecutive patients with ALC from the same center prospectively recruited between November 2005 and September 2006 were added to the 100 Czech patients included in the pilot study, and

Table 4 Association of the [*IL1RN**2/*2; *IL1B* –31T+] diplotype with ALC in patients with ALC, healthy population controls and heavy drinkers without cirrhosis.

Population	Unadjusted		Adjusted for age	
	p	OR (95% CI)	p	OR (95% CI)
Pilot study (100 Czech ALC patients vs. 180 Czech population controls)	0.006	8.80 (1.86–41.6)	0.005	9.45 (1.96–45.7)
Extended pilot study (198 Czech ALC patients vs. 370 Czech population controls)	0.083	2.09 (0.97–4.84)	0.092	2.06 (0.89–4.78)
Validation I (173 German ALC patients vs. 331 German population controls)	0.973	0.99 (0.52–1.90)	[a]	[a]
Validation II (173 German ALC patients vs. 109 German heavy drinkers without ALC)	0.326	1.63 (0.61–4.34)	0.484	1.44 (0.52–3.95)

[a]As there was no age difference between cases and controls, no adjustment for age was performed. OR, odds ratio; CI, confidence interval; ALC, alcoholic liver cirrhosis.

the control sample was extended by 190 subjects from the MONICA study. Thus, the total population sample comprised 198 patients and 370 controls. The association of the [*IL1RN**2/*2; *IL1B* –31T+] diplotype was not significant; however, it was close (age-adjusted OR 2.06, 95% CI 0.89, 4.78, p=0.09). The non-significance could be caused by increased prevalence of the diplotype in the extended number of controls (2.8%).

Second, the [*IL1RN**2/*2; *IL1B* –31T+] diplotype frequency of German ALC patients was compared to that of the German population control group. Assuming an OR of 2.5, a required sample size of 173 cases and 331 controls was used in the validation study. No association with ALC due to the same prevalence of the diplotype in patients and controls (8.8% in both groups) was found.

Finally, the association was further tested in German patients with ALC and heavy drinkers without liver disease as controls. Even then, no significant association was found (see Table 4), and the frequency of the [*IL1RN**2/*2; *IL1B* –31T+] diplotype in the group of healthy heavy drinkers was not significantly different from that in German population controls (5.5% vs. 8.8%, p=0.30).

Discussion

In our study, we included only those genes encoding cytokines whose role in ALC was proved in animal models and those polymorphisms whose impact on gene expression or protein function was described in in vitro studies. Moreover, the included polymorphisms have been associated with an increased risk of alcoholic liver disease (26, 29, 30, 35), accelerated progression of fibrosis in chronic hepatitis B (36) and endotoxin hyporesponsiveness in humans (21). To include gene variants with the largest possible effect size, we also investigated the [*IL1RN**2/*2; *IL1B* –31T+] diplotype, because it increases the production of IL-1β more than either of the polymorphisms alone (24, 25). This is enabled by the low strength of LD which permits the additive effect of both proinflammatory alleles (i.e., *IL1RN**2 and *IL1B* –31T) on IL-1β production. Importantly, both polymorphisms increased the risk of ALC in the Asian population with a high OR for the disease exceeding 4.5 (27, 28). Contrary to the paper by Takamatsu et al. (27), we did not include the *IL1B* +3953C/T polymorphism because it does not alter in vitro protein secretion (37), and we did not include the silent *IL1B* –511C allele. Instead, we analyzed the *IL1B* –31T allele, which is in complete LD with the *IL1B* –511C allele, creates a TATAA sequence in the promoter and results in a five-fold increase of *IL1B* transcription (24).

Selection of the appropriate cases and controls is a major issue in genetic studies on ALC. In case-control studies, individuals with the disease under investigation (cases) are compared to individuals who do not have the disease, but who are thought to be comparable in other respects (controls). Inclusion of patients with advanced ALC reduces the risk of misclassification, increases phenotypic homogeneity and the power to detect genetic association. Indeed, selection of extreme cases, such as patients undergoing liver transplantation, further increases the power of the study but also inflates the estimation of population-based parameters, such as population attributable risk. However, it was not the purpose of our study to assess this measure because such assessment would require a significantly higher number of individuals with ALC recruited in a population-based manner.

Daly and Day (38) advocated that control individuals in genetic studies should reveal a comparable exposure to alcohol as cases. Compared to population controls, use of ''hypernormal'' controls (healthy heavy drinkers) would be expected to improve the power by increasing the difference in susceptibility allele frequency between cases and controls. However, this benefit is usually not substantial and is counterweighted by the costs and effort of defining a ''hypernormal'' population (39). In our study, investigating healthy German heavy drinkers did not yield a positive association with an increased risk of ALC and the frequency of the [*IL1RN**2/*2; *IL1B* –31T+] diplotype in this group was not significantly different from that in German population controls. Taken together, it does not seem to us that inclusion of ''hypernormal'' controls is superior to population controls.

The discrepancy between our findings and the previously published studies (26–30, 35, 40) might be explained by ethnical differences and/or the lack of statistical power. Moreover, none of the studies (26–30, 35, 40) included an independent validation sample which should be implemented particularly in smaller studies that are likely to be subject to a type 1 error which overestimates a genetic effect due to chance (41). Verifying positive findings in an independent validation sample helps to detect false associations, as exemplified in our study in which a seemingly positive association from a pilot study was rejected by its subsequent validation in an additional group of subjects with the same phenotype.

Also, genetic association studies are frequently subject to type 2 errors (false negativity), because polymorphisms are not causal and act only as phenotype modifiers. In our study, we maximized the power to detect an association with an increased risk of ALC by (i) selecting genes and polymorphisms that are expected to have the highest possible effect size (protection against alcohol-induced liver injury in animal knockout models, additive effect of *IL1B* –31T and *IL1RN*2* alleles on the secretion rate of IL-1β), (ii) inclusion of patients with advanced ALC, and (iii) inclusion of ''hypernormal'' controls. The design of our study would enable us to detect biologically significant associations if they exist. Our study was not powered enough to detect OR below 2, although polymorphisms with a small effect size could act as disease modifiers and confer a measurable attributable risk. However, their clinical significance is questionable. In fact, they could hardly be used for predicting the risk of ALC in ethanol-drinking individuals, which

is the ultimate goal of identifying disease susceptibility markers.

In conclusion, although there is little doubt that cytokine mediated immune reactions do play a role in the pathogenesis of ALC, hereditary susceptibility caused by variants in the corresponding genes seems to be low, or at least such is the case in a CEU population.

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Hepatocyte-specific IRF3 and Type I interferons are protective in alcohol-induced liver injury in mice via cross-talk with macrophages

Short title: Hepatocytes control inflammatory response in alcohol-induced liver injury

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List of abbreviations: ALD, alcoholic liver disease; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-alpha; TLR, toll-like receptor; IFN, interferon; MyD88, myeloid-differentiation factor 88; NF-κB, nuclear factor κB; TRIF, TIR domain-containing adaptor inducing interferon-beta; TBK1/IKKε, TANK-binding kinase 1/inhibitor of κB kinase epsilon; IRF3, interferon regulatory factor-3; KO, knock-out; IFNAR, Type I interferon α/β receptor; BM, bone-marrow; IL-1β, interleukin-1 beta; IL-10, interleukin 10; LMNC, liver mononuclear cells, PBMC, peripheral blood mononuclear cells; WT, wild-type; ISG, interferon stimulated gene; IL-10R, interleukin 10 receptor.

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Abstract

Alcoholic liver disease (ALD) features increased hepatic exposure to bacterial lipopolysaccharide (LPS). Toll-like receptor-4 (TLR4) recognizes LPS and activates signaling pathways depending on MyD88 or TRIF adaptors. We previously showed that MyD88 is dispensable in ALD. TLR4 induces Type-I interferons (IFN) in MyD88-independent manner that involves interferon regulatory factor-3 (IRF3). We fed alcohol or control diets to wild-type and IRF3 knock-out (KO) mice, and to mice with IRF3 deficiency in hepatocytes. Whole-body IRF3-KO, but not wild-type, mice were protected from alcohol-induced liver injury, steatosis and inflammation. In contrast, deficiency of IRF3 only in parenchymal cells rendered the mice more susceptible to alcohol-induced liver injury, associated with higher pro-inflammatory cytokine TNF-α, lower anti-inflammatory cytokine IL-10 and lower Type-I IFN production compared to wild-type mice. Co-culture of wild-type primary murine hepatocytes with liver mononuclear cells (LMNC) resulted in higher LPS-induced IL-10, higher IFN-β, and lower TNF-α levels compared to LMNC alone. Type-I IFN was important since co-cultures of hepatocytes with LMNC from Type-I IFN receptor KO mice showed attenuated IL-10 levels compared to control co-cultures from wild-type mice. We further identified that Type-I IFNs potentiated LPS-induced IL-10 and inhibited inflammatory cytokine (TNF-α) production in both RAW264.7 murine macrophages and human leucocytes, indicating preserved cross-species effect. These findings suggest that hepatocytes produce type-I IFN in TLR4/IRF3-dependent manner. Further, hepatocyte-derived type-I IFNs increase anti-inflammatory and suppress pro-inflammatory cytokines production by liver macrophages in a paracrine manner. *In conclusion*, our results indicate that hepatocyte-specific IRF3 activation and resulting type I IFNs have protective effects

in ALD via modulation of pro- and anti-inflammatory functions in macrophages. These results suggest potential therapeutical targets in ALD.

Alcoholic liver disease (ALD) is the most common drug abuse-induced liver disease and accounts for 40% of deaths from cirrhosis in the United States (1). Gut-derived lipopolysaccharide (LPS), a component of the gram-negative bacterial wall, has been proposed as a key player in the pathogenesis of ALD (2, 3). Exposure to LPS during chronic alcohol consumption results in increased production of inflammatory mediators, leading to progression of liver injury (4). Indeed, mice treated with antibiotics to eliminate gut microflora, or mice deficient in tumor necrosis factor-alpha (TNF) type I receptor were protected from alcohol-induced liver injury (5, 6).

Recognition of pathogen-derived molecules occurs through pattern recognition receptors such as Toll-like receptors (TLR), which are widely expressed on parenchymal and non-parenchymal cell types in the liver (7). TLR4 recognizes LPS and activates two signaling pathways via recruitment of adaptor molecules (8, 9). Recruitment of the common TLR adaptor, myeloid differentiation factor 88 (MyD88), leads to rapid activation of nuclear factor B (NF-κB) and increased TNFα production, while recruitment of TIR domain-containing adaptor inducing interferon-beta (TRIF) activates TANK-binding kinase 1/inhibitor of κB kinase epsilon (TBK1/IKKε) and interferon regulatory factor 3 (IRF3), leading to production of type I interferons (IFNs) and delayed NF-κB activation (10-12).

We have previously reported that MyD88 deficiency failed to prevent alcohol-induced liver damage and inflammation suggesting that TLR4-mediated MyD88-independent pathways are important in induction of ALD (13). The significance of MyD88-independent pathways including activation of IRF3 in ALD is yet to be evaluated.

Considering the importance of LPS-induced inflammatory activation in ALD (3) and the role of MyD88-independent downstream pathways in TLR4 signaling (13), we hypothesized that IRF3 was critical in alcohol-induced liver injury. Given the differential input of parenchymal and non-parenchymal cells in pathophysiology of ALD, we further hypothesized that IRF3 may be critical in alcoholic liver injury in a cell-specific manner. Therefore, we employed a chimeric mouse model to evaluate the effect of chronic alcohol feeding on liver damage, steatosis and inflammation in animals with selective deficiency of IRF3 in liver parenchymal cells.

Here we demonstrate that hepatocyte-specific IRF3 activation and downstream type I IFN induction have protective effects in ALD. We report that disruption of IRF3 in liver parenchymal cells decreases type I IFN production and increases liver injury due to dysregulated expression of pro- and anti-inflammatory cytokines.

Materials and methods

Animal studies

All animals received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School. Six to eight-week-old, female C57Bl/6 wild-type, IRF3-deficient (IRF3-KO) and Type I interferon α/β receptor 1-deficient (IFNAR-KO) mice (kind gift of Jonathan Sprent, Scripps Research Institute, La Jolla, CA), were employed. Some animals were fed with the Lieber-DeCarli diet (Dyets, Inc., Bethlehem, PA) with 5% (vol/vol) ethanol (36% ethanol-derived calories) for 4 weeks; pair-fed control mice matched the alcohol-derived calories with dextran-maltose (13). Chimeric mice were generated by transplanting wild-type (C57Bl/6) bone marrow into irradiated, IRF3 deficient mice (IRF3-KO/WT-BM). Serum was stored at -80°C. Livers were snap-frozen in liquid nitrogen for proteins, or stored in RNAlater (Qiagen GmbH, Hilden, Germany) for RNA extraction, or fixed in 10% neutral-buffered formalin for histopathological analysis.

Biochemical assays

Serum alanine aminotransferase (ALT) was determined using a kinetic method (D-Tek LLC., Bensalem, PA). Liver triglyceride levels were assessed using the L-Type Triglyceride H kit (Wako Chemicals USA Inc., VA).

Cytokine measurement

Mouse IL-1β ELISA kit was purchased from R&D (R&D systems, Inc., Minneapolis, MN), mouse and human TNF-α, IL-1β and IL-10 kits from BD Bioscience (BD Biosciences, San Jose, CA) and mouse IFN-β kit from PBL (PBL interferon source, Piscataway, NJ).

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). SybrGreen-based real-time quantitative polymerase chain reaction was performed using the iCycler (Bio-Rad Laboratories Inc., Hercules, CA), as described (13); primer sequences are shown in Table 1.

Histopathological analysis

Sections of formalin-fixed livers were stained with hematoxylin and eosin and analyzed by microscopy.

Isolation of hepatocytes and liver mononuclear cells

Animals received anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg); the livers were perfused with saline solution followed by *in vivo* digestion, as we previously described (13). The hepatocytes were separated by centrifugation, liver mononuclear cells (LMNCs) were purified by centrifugation in Percoll gradient.

Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were separated from blood of healthy volunteers by centrifugation in Ficoll gradient, as we previously described (14).

In vitro experiments

Primary hepatocytes and LMNCs were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% insulin, transferrin, selenium (ITS) solution. Primary hepatocytes were seeded in 6-well collagen-coated plates, LMNCs (10^6/insert) were plated in cell-culture inserts with pore diameter 0.4 µm (Becton Dickinson Labware, Bedford, MA). Hepatocytes, LMNCs or co-cultures of hepatocytes + LMNCs were stimulated with LPS (Sigma, St. Louis, MO). IFN-β, IL-10 and TNF-α were measured in supernatants using ELISA.

RAW264.7 macrophages were stimulated with LPS, recombinant mouse IFN-α2a (eBioscience, San Diego, CA), recombinant mouse IL-10 (Peprotech Inc., Rocky Hill, NJ) or with anti-mouse IL-10 receptor antibody (Biolegend, San Diego, CA).

Human PBMCs were stimulated with LPS, recombinant human IFN-α (PBL, Piscataway, NJ), recombinant IL-10 (Ebioscience, San Diego, CA) or IL-10 receptor antibody (R&D systems, Inc., Minneapolis, MN).

Statistical Analysis

Statistical significance was determined using the T-test or the nonparametric Kruskal-Wallis test. Regression plots were constructed using the Graphpad Prism 5.01 (GraphPad software, Inc., La Jolla, CA). Data are shown as mean ± standard error of the mean (SEM) and were considered statistically significant at $P < 0.05$.

Results

IRF3-deficiency protects against alcohol-induced liver damage

TLR4 recognizes LPS and activates two signaling pathways by utilizing the adaptor molecules MyD88 or TRIF, respectively. We showed that MyD88 is dispensable in ALD (13). In addition to induction of inflammatory cytokines via NF-κB, MyD88-independent activation of TLR4 triggers production of Type I IFNs, which is largely dependent on activation of intracellular pathways involving interferon regulatory factor-3 (IRF3) (12). To define the importance of the MyD88-independent, IRF3-dependent signaling cascade and Type I IFNs in alcohol-induced liver injury, we fed ethanol or isocaloric control (pair feeding) diet to wild-type (WT) and IRF3-KO mice.

Histopathological analysis revealed that chronic alcohol feeding induced micro- and macrovesicular steatosis and inflammatory cell recruitment in ethanol-fed wt mice, suggestive of ALD (Fig 1A). In contrast, none of the histopathological features of ALD were observed in IRF3-KO mice (Fig. 1A). Consistent with the histopathology, serum ALT levels were significantly higher in alcohol-fed WT mice, but not in the IRF3-KO mice, compared to the pair-fed controls (Fig. 1B). We also found that the expression of inflammatory cytokines TNF-α and IL-6 in the liver was significantly higher in alcohol-fed WT mice compared to pair-fed controls; this alcohol-induced pro-inflammatory state was absent in IRF3-KO mice (Fig. 1C,D). Alcohol feeding to WT mice triggered expression of Type I IFN stimulated gene (ISG) 56, suggesting activation of Type I IFN signaling in alcohol-induced liver injury. In contrast, alcohol feeding of IRF3KO mice failed to upregulate ISG56 (Fig. 1E). These data suggested a role of IRF3 and/or Type I IFNs in alcohol-induced liver injury.

Hepatocyte-specific IRF3 deficiency aggravates alcohol-induced liver damage

The liver functions with a complex co-existence of parenchymal (hepatocytes) and non-parenchymal cells. To explore whether the protective effect of IRF3 in alcoholic liver injury is mediated by hepatocytes or (BM)-derived immune cells, we generated IRF3-chimeric mice by transplanting wild-type BM into irradiated, IRF3-deficient mice (IRF3-KO/WT-BM mice). Wild-type mice with wild-type bone marrow transplant served as controls (WT/WT-BM). As expected, WT/WT-BM mice developed ALD after 4 weeks of Lieber-DiCarli diet, as indicated by liver steatosis, inflammatory infiltrate and liver injury, compared to pair-fed controls (Fig. 2A,B,C).

In sharp contrast to WT/WT-BM mice, IRF3-KO/WT-BM mice showed aggravation of alcohol-induced liver injury, as indicated by exaggerated steatosis and inflammatory infiltrate on histology (Fig. 2A). This finding was accompanied by elevation in serum ALT and in liver triglycerides, compared to WT/WT-BM ethanol-fed mice (Fig. 2B,C). Further, IRF3-KO/WT-BM mice showed exaggerated expression of inflammatory cytokines TNF-α, IL-6, IL-1β (Fig. 2D-G). These data suggested a protective role of hepatocyte-specific IRF3 in ALD by limiting liver inflammation and injury.

Hepatocyte-specific IRF3 deficiency is associated with decreased Type I IFN and IL-10 induction

Activation of IRF-3 leads to preferential induction of IFN-β (15). We identified that, in contrast to WT mice, IRF3-KO/WT-BM mice showed a significantly decreased expression of IFN-β (Fig. 3A). This finding indicated that aggravated liver injury in IRF3-KO/WT-BM mice is associated

with deficient induction of the IRF3-dependent type I IFNs and suggested possible involvement of IRF3- and Type I IFN-dependent anti-inflammatory factors in alcohol-induced liver injury. We thus analyzed the expression of the Type I IFN-dependent gene, IL-10, which is a major anti-inflammatory cytokine (16). Liver IL-10 mRNA was substantially upregulated by alcohol-feeding in WT/WT-BM mice, but not in IRF3-KO/WT-BM mice (Fig. 3B), and IL-10 protein levels were significantly lower in ethanol-fed IRF3-KO/WT-BM mice compared to controls (Fig. 3C). Collectively, these findings suggested that hepatocyte-specific IRF3 is required for expression of IFN-β and IL-10 in alcohol-induced liver injury.

Hepatocytes produce Type I IFN in IRF3-dependent manner and modulate cytokine production in liver mononuclear cells

Our findings suggested that expression of liver IL-10 is linked to activation of hepatocyte-specific IRF3. We thus intended to dissect the cell-specific role in the IRF3-dependent type I IFNs vs IL-10 balance during ALD. We found that isolated primary hepatocytes of WT, but not those of IRF3-KO mice, produced IFN-β in response to LPS (Fig. 4 A,B). Both unstimulated and LPS-stimulated WT hepatocytes produced significantly more IFN-β than LMNCs (Fig. 5A), suggesting that hepatocytes are a dominant source of IFN-β in the liver. On the contrary, IL-10 was produced mainly by LMNCs, which supports the data that Kupffer cells stimulated with LPS produce IL-10 (17, 18). Importantly, LMNCs co-culture with primary hepatocytes resulted in increased IL-10 production, compared to either cell types alone, which was further significantly increased upon stimulation with LPS (Fig. 5B). The induction of IL-10 in hepatocyte/LMNC co-culture exceeded a merely additive contribution of both cell types to the secretion of IL-10, suggesting that hepatocyte-derived IFN-β facilitates the production of IL-10 in immune cells in

the liver. In contrast, there was no IL-10 induced by co-cultures of hepatocytes and LMNCs from IFNAR-KO mice (Fig. 5B), supporting our hypothesis that hepatocyte-dependent enhancement of IL-10 expression in LMNCs is a Type I IFN-dependent process.

Given the tight control of the pro-and anti-inflammatory balance in the liver we further asked whether Type I IFN-dependent IL-10 production may affect the level of TNF-α in liver immune cells. We identified that TNF-α production by WT LMNCs was significantly downregulated upon their co-culture with WT hepatocytes; such effect was absent in LMNCs of IFNAR-KO mice (Fig. 5C).

To further evaluate if regulation of IL-10 by Type I IFNs is preserved across species, we stimulated murine RAW264.7 murine macrophages or human peripheral blood mononuclear cells (PBMC) with LPS and Type I IFN, and identified a significant increase of IL-10 in the presence of Type I IFN compared to stimulation with LPS alone in both species (Fig. 6A,B). We found that expression of TNF-α was significantly decreased in RAW264.7 murine macrophages stimulated with LPS in the presence of recombinant IL-10, whereas neutralization of the IL-10 receptor (IL-10R) with anti-IL10R antibody significantly upregulated TNF-α (Fig. 6C). We also observed a dose-dependent inhibition of TNF-α and IL-1β by IL-10 in human PBMCs (Fig. 6D). Further, a 50% inhibitory concentration (IC_{50}) of IL-10 caused a significant inhibition of LPS-triggered TNF-α and IL-1β (Fig. 6D,E,F), while inhibition of IL-10 receptors using IL-10R Ab significantly upregulated secretion of inflammatory cytokines in human PBMCs (Fig. 6E,F). These data confirmed that IL-10 is upregulated by Type I IFNs across species.

Taken together, these data demonstrate that hepatocyte-derived Type I IFNs upregulate IL-10 and downregulate inflammatory cytokines in non-parenchymal cells in the liver. More importantly, they outline the paradigm of intercellular cooperation and regulation in the liver, where hepatocytes control the inflammatory potential of immune cells.

Discussion

Chronic consumption of ethanol is tightly linked to liver steatosis and inflammation in human disease as well as in experimental models. Whereas activation of TLR4-dependent pathways by gut-derived LPS and induction of inflammatory cytokines has been traditionally attributed to BM-derived Kupffer cells (19), the role of crosstalk between parenchymal (i.e. hepatocytes) and non-parenchymal (BM-derived immune cells) in ALD remains elusive. Here we demonstrate that liver response to insults is a multistep process: hepatocyte-specific IRF3 drives Type I IFN induction in the liver and hepatocyte-derived Type I IFN leads to a modulation of inflammatory cytokines in non-parenchymal BM-derived cells (Fig. 7). Our novel findings outline a link between hepatocytes and liver immune cells in modulation of innate immune signaling in ALD.

BM-derived cells are considered to be the main targets of pathogen-derived products in the liver due to their strategical position to encounter pathogens in the portal system and broad-range expression of TLRs. Our study provides novel lines of evidence that hepatocytes are the main producers of Type I IFNs in response to alcohol/LPS exposure. First, chimeric mice containing IRF3-deficient hepatocytes and wild-type BM-derived cells show a similar reduction in baseline and ethanol-induced expression of Type I IFNs as mice deficient in IRF3 throughout the body (IRF3-KO mice). Second, *ex vivo* stimulation of wild-type primary mouse hepatocytes with LPS resulted in a significant upregulation of Type I IFNs, in contrast to hepatocytes isolated from IRF3KO mice that failed to induce Type I IFNs.

The liver is a main target of intestinally-derived bacterial products and various models of ALD have shown protective phenotype in mice deficient in TLR4, CD14 receptor and LPS-binding protein (20-22), rendering LPS a likely candidate mediator of TLR4-dependent activation of

IRF3 and induction of Type I IFNs. Accordingly, we found induction of Type I IFNs in livers of mice fed with ethanol as well as in primary mouse hepatocytes stimulated with LPS. Moreover, deficiency of IRF3 abrogated Type I IFN induction and signaling in liver, suggesting that IRF3 is a dominant signaling molecule inducing Type I IFNs in ALD.

Our study defines induction of Type I IFNs via IRF3 in hepatocytes and downregulation of inflammatory cytokines in BM–derived cells as two complementary, yet independent mechanisms by which TLR4 controls the extent of alcohol-induced liver inflammation and injury. Kupffer cells stimulated via TLR4 are a main source of inflammatory cytokines in the liver and promote tissue inflammation, injury and fibrosis (19). Thus, TLR4 seems to activate IRF3 in both parenchymal and non-parenchymal liver cells: here we demonstrate that while the signaling pathways are shared, we observed a cell-specific response to LPS, with a distinct outcome. While BM-derived cell-specific IRF3 is instrumental in activation of NF-κB and induction of inflammatory cytokines, thereby playing a direct proinflammatory role (10, 23, 24), hepatocyte-specific IRF3 seems to dampen TLR4-induced inflammatory response by indirect (paracrine) mechanism mediated by Type I IFNs. The importance of this cell-specific activation of IRF3 and Type I IFNs is emphasized by our finding that aggravated liver inflammation and injury was observed in mice chimeras lacking IRF3 in hepatocytes, and was further associated with a significantly decreased expression of IL-10, a major anti-inflammatory cytokine, in the liver.

Our finding of Type I IFN-dependent induction of the anti-inflammatory state in the liver is supported by the fact that the IL-10 promoter contains a Type I IFN-dependent responsive element (25) which makes this cytokine a Type I IFN-dependent anti-inflammatory mediator.

We found that, *ex vivo*, liver mononuclear cells synthesized significantly more IL-10 when co-cultured with primary hepatocytes that produced significant amounts of Type I IFNs. The existence of a hepatocyte/immune cell regulation loop is further supported by our finding that the facilitation of IL-10 production by hepatocyte-specific Type I IFNs in liver mononuclear cells was abrogated in cells lacking Type I IFN receptor, and that administration of recombinant Type I IFNs significantly upregulated IL-10 in wild-type mononuclear cells. Furthermore, our data show that administration of IL-10 to mouse macrophages or human PBMCs stimulated with LPS significantly suppresses inflammatory cytokines, and therefore support the critical role of IL-10 in determining the pro- and anti-inflammatory balance in the pathogenesis of ALD (17, 26). Taken together, these findings demonstrate that full expression of anti-inflamatory factors in BM-derived cells is dependent on Type I IFN signaling from hepatocytes, which is regulated by IRF3.

TLRs fulfill a variety of functions in the liver, and inhibition of TLR4 signaling may alter biological processes related to liver inflammation, injury and fibrosis (20-22, 27). TLR4 also promotes disease progression in alcoholic and nonalcoholic steatohepatitis (13, 28), primary sclerosing cholangitis (29) and ischemia-reperfusion injury (30), and therefore represents a potential therapeutic target. Indeed, use of probiotics, antifibrotics or anti-inflammatory agents are proposed as potential therapeutic options for these diseases (31). However, excessive TLR signaling triggers not only harmful responses, but also beneficial responses, such as clearance of microorganisms (32), tissue regeneration (33), and, as we demonstrate in this study, indirect induction of anti-inflammatory loop via induction of protective Type I IFNs in hepatocytes in IRF3-dependent manner. Therefore, it seems plausible that fine tuning, in contrast to approaches

that would completely abrogate TLR signaling, may have a future in efforts to translate TLR pathophysiology into clinical practice in human liver diseases.

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Tables

Table 1. Real-time PCR primers.

Target gene	**Forward primer (5’→ 3’)**	**Reverse primer (5’→ 3’)**
18S	gta acc cgt tga acc cca tt	cca tcc aat cgg tag tag cg
TNF-A	cac cac cat caa gga ctc aa	agg caa cct gac cac tct cc
IL-6	aca acc acg gcc ttc cct act t	cac gat ttc cca gag aac atg tg
IL-1B	tct ttg aag ttg acg gac cc	tga gtg ata ctg cct gcc tg
IL-10	ctg gac aac ata ctg cta acc g	ggg cat cac ttc tac cag gta a
IFN-B	agc tcc aag aaa gga cga aca t	gcc ctg tag gtg agg gtt gat ct
ISG-56	ggg cct tgc agg cat cac ctt	tcc tgc ctt ctg ggc tgc ct

Figure legends

Fig. 1. *IRF3-deficiency protects against alcohol-induced liver damage.*

Wild-type (WT) and IRF3-deficient (IRF3-KO) were fed Lieber DeCarli ethanol or control (pair-fed) diet and sacrificed after 4 weeks. Livers were fixed in formalin and stained with H&E; magnification 200x (A). Serum ALT levels (B) were analyzed. Messenger RNA levels of liver (C) tumor necrosis factor α (TNFA), (D) interleukin 6 (IL-6) and (E) interferon stimulated gene ISG56 were analyzed by real-time PCR and normalized to 18s.Values are shown as mean ± SEM fold increase over WT pair-fed control group (3-6 mice per group). Numbers in graphs denote p values; *) $p < 0.05$ vs. pair-fed WT mice; #) $p < 0.05$ vs. ethanol-fed WT mice.

Fig. 2. *Hepatocyte-specific IRF3 deficiency aggravates alcohol-induced liver damage*

Wild-type mice with transplanted WT bone marrow (WT/WT-BM) and IRF3-deficient mice with transplanted wild-type bone marrow (IRF3-KO/WT-BM) were fed Lieber DeCarli ethanol or control (pair-fed) diet and sacrificed after 4 weeks. Livers were fixed in formalin and stained with H&E; magnification 200x, arrows point at inflammatory foci (A). Serum ALT levels (B) and liver triglycerides (C) were analyzed. Messenger RNA levels of liver (D) tumor necrosis factor α (TNFA), (E) interleukin 6 (IL-6) and (F) interleukin-1β (IL-1β) were analyzed by real-time PCR and normalized to 18s. Liver IL-1β levels were analyzed using ELISA (G). Values are shown as mean ± SEM fold increase over wild-type pair-fed control group (5-7 mice per group). Numbers in graphs denote p values; *) $p < 0.05$ vs. pair-fed wild-type mice; #) $p < 0.05$ vs. ethanol-fed wild-type mice.

Fig. 3. *Hepatocyte-specific IRF3 deficiency is associated with decreased Type I IFN and IL-10 induction*

Wild-type mice with transplanted WT bone marrow (WT/WT-BM) and IRF3-deficient mice with transplanted wild-type bone marrow (IRF3-KO/WT-BM) were fed Lieber DeCarli ethanol or control (pair-fed) diet and sacrificed after 4 weeks. Messenger RNA levels of (A) liver interferon β (IFN-β) and (B) interleukin 10 (IL-10) were analyzed by real-time PCR and normalized to 18s. Liver IL-10 protein levels were analyzed using immunoblot analysis (C). Values are shown as mean ± SEM fold increase over wild-type pair-fed control group (5-7 mice per group). Numbers in graphs denote p values. *) $p < 0.05$ vs. pair-fed wild-type mice; #) $p < 0.05$ vs. ethanol-fed wild-type mice.

Fig. 4. *Hepatocytes induce Type I IFN in IRF3-dependent manner.*

Primary hepatocytes were isolated from WT and IRF3-deficient (IRF3-KO) mice and stimulated with 100 ng/mL lipopolysaccharide (LPS). Messenger RNA levels of interferon β (IFN-β) were analyzed by real-time PCR and normalized to 18s (A). IFN-β protein levels in supernatant were analyzed using ELISA (B). Values are shown as mean ± SEM (5 mice per group). Numbers in graphs denote p values; *) $p < 0.05$ vs. nonstimulated WT hepatocytes; #) $p < 0.05$ vs. LPS-stimulated WT hepatocytes.

Fig. 5. *Hepatocyte-derived Type I IFNs upregulate IL-10 and downregulate TNF-α in liver mononuclear cells*

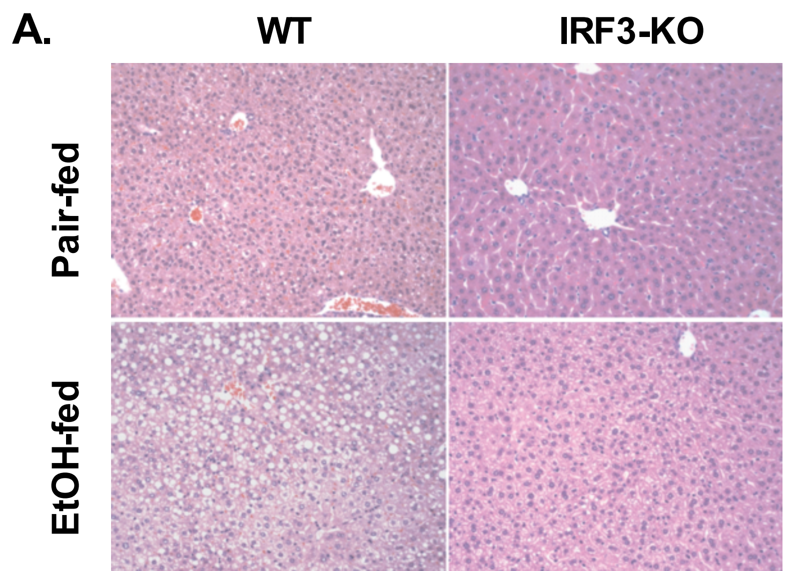
Primary hepatocytes and liver mononuclear cells (LMNC) were isolated from WT or Type I interferon α/β receptor 1-deficient (IFNAR-KO) mice and stimulated with 100 ng/mL lipopolysaccharide (LPS) in transwell cell-culture systems as indicated. Protein levels of (A) interferon β (IFN-β), (B) interleukin 10 (IL-10) and (C) tumor necrosis factor α (TNF-α) in cell-free supernatants were analyzed using specific ELISA. Values are shown as mean ± SEM (5 mice per group). Numbers in graphs denote p values. *) $p < 0.05$ vs. nonstimulated WT hepatocytes; #) $p < 0.05$ vs. LPS-stimulated WT hepatocytes.

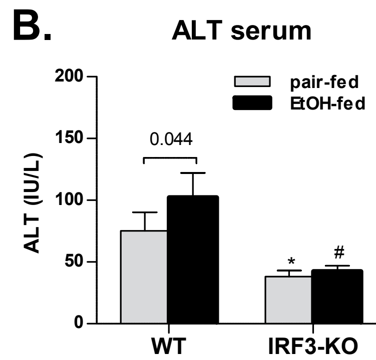
Fig. 6. *Type I IFN-dependent induction of IL-10 modulates inflammatory cytokines in mononuclear cells*

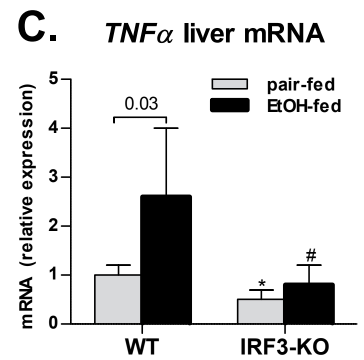
Murine RAW264.7 macrophages were stimulated with 100 ng/mL LPS, 1000 IU/mL murine recombinant IFNα2a, 10 ng/mL recombinant murine IL-10 and 1 µg/mL anti-mouse IL10 receptor antibody (anti IL-10R Ab). Human peripheral blood mononuclear cells (PBMCs, N=4) were stimulated with 10 ng/mL LPS, 1000 IU/mL human recombinant IFNα2, 10-2000 pg/mL recombinant human IL-10 and 1 µg/mL anti-human IL10 receptor antibody (anti IL-10R Ab). Protein levels of (A,B) interleukin 10 (IL-10), (C,D,E) tumor necrosis factor α (TNF-α) and (D,F) interleukin 1-β (IL-1β) were analyzed using ELISA. Values are shown as mean ± SEM. Numbers in graphs denote p values; *,#,§,†) $p < 0.05$ vs. respective control group

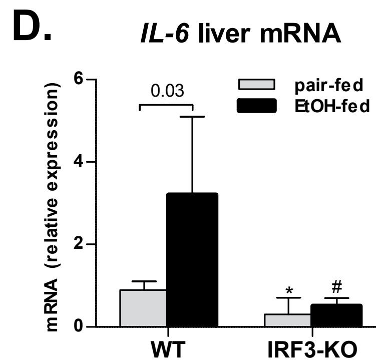
Fig. 7. *Proposed mechanism of hepatocyte-mediated control of inflammatory responses in alcohol-induced liver injury***.**

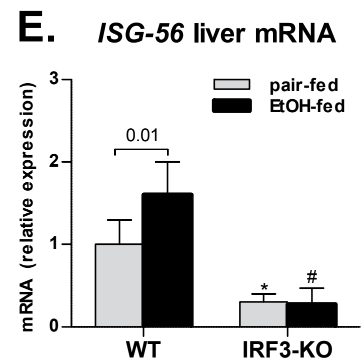
Chronic alcohol consumption results in increased exposure of the liver to the gut-derived lipopolysaccharide (LPS). LPS is recognized via the Toll-like receptor 4 (TLR-4) on non-parenchymal and parenchymal cells. In non-parenchymal cells, LPS increases production of TNF-α. In parenchymal cells, LPS induces Type I interferons (IFN) in IRF3-dependent manner. In turn, hepatocyte-derived Type I IFNs enhance IL-10 and downregulate TNF-α in non-parenchymal cells, thus regulating the balance between inflammatory and anti-inflammatory cytokines in the liver.











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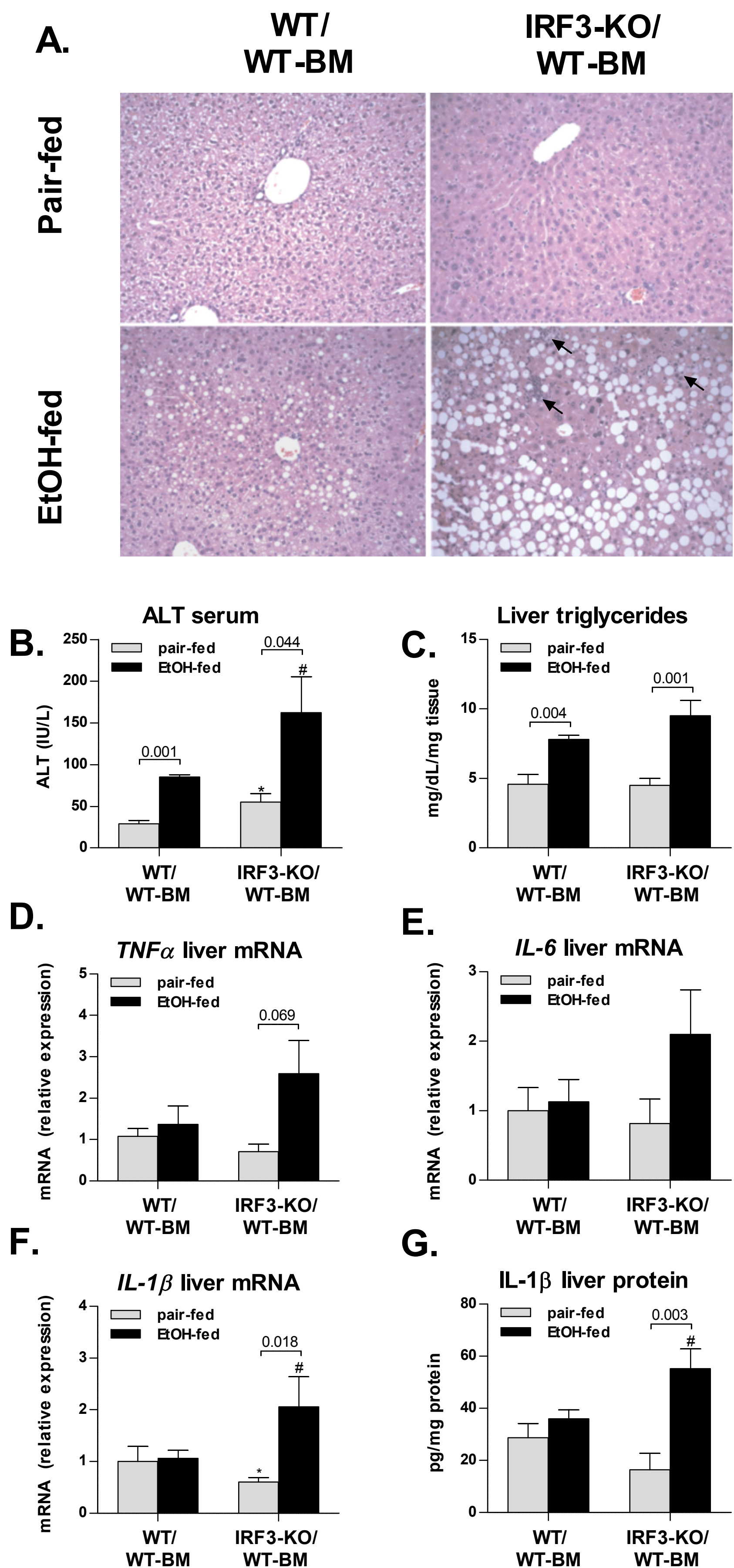

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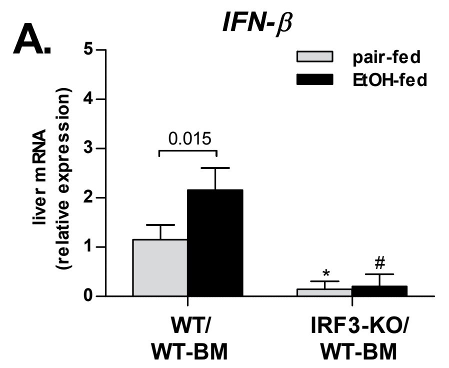
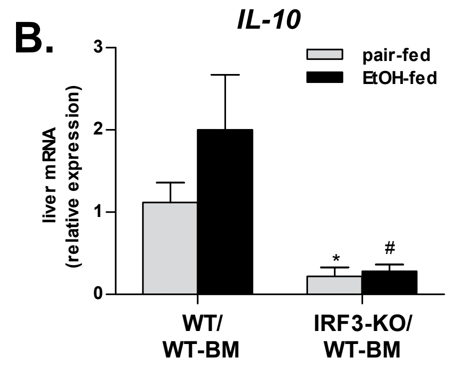
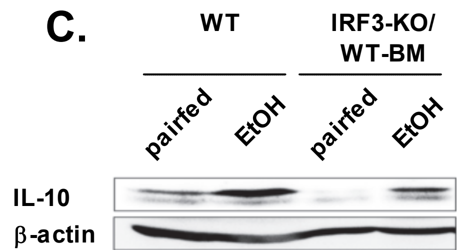






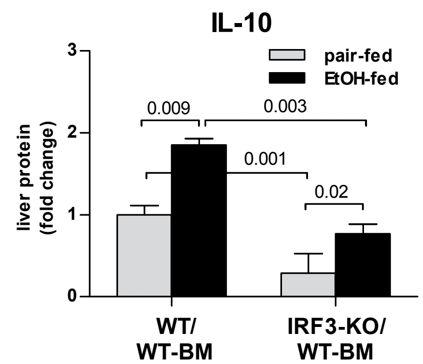
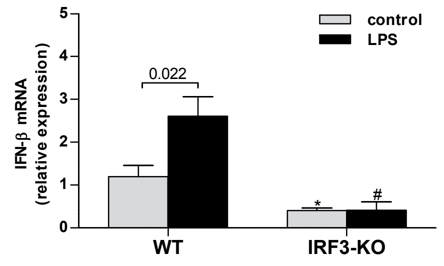
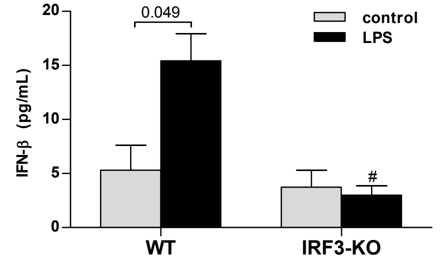


Figure 3.

Hepatocytes





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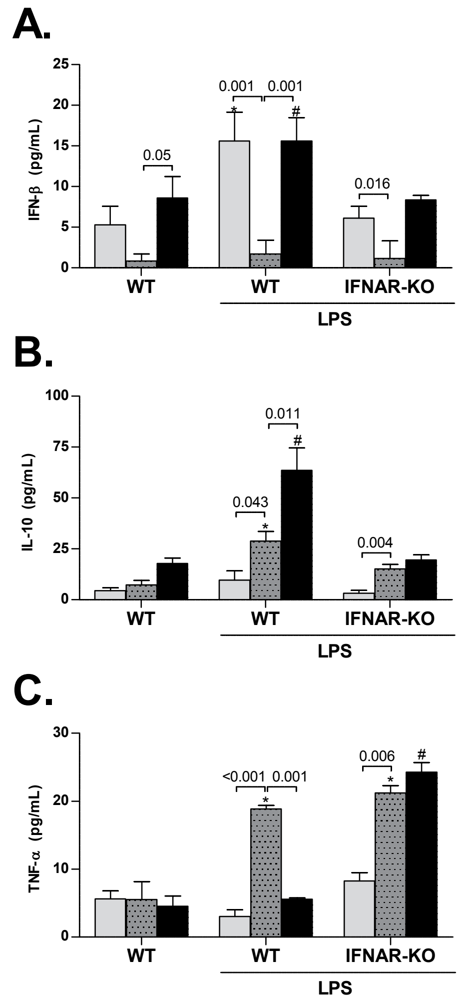

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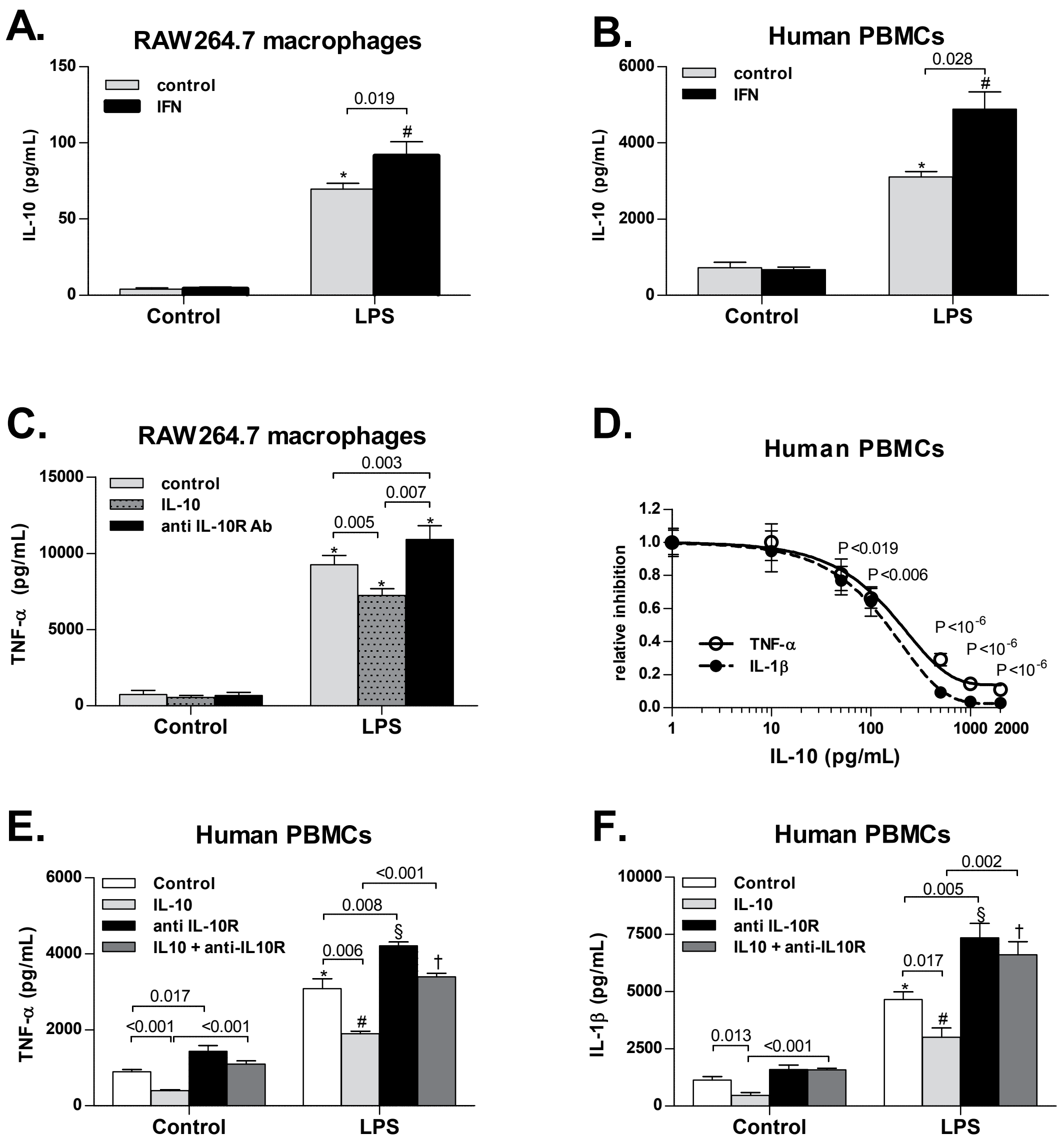

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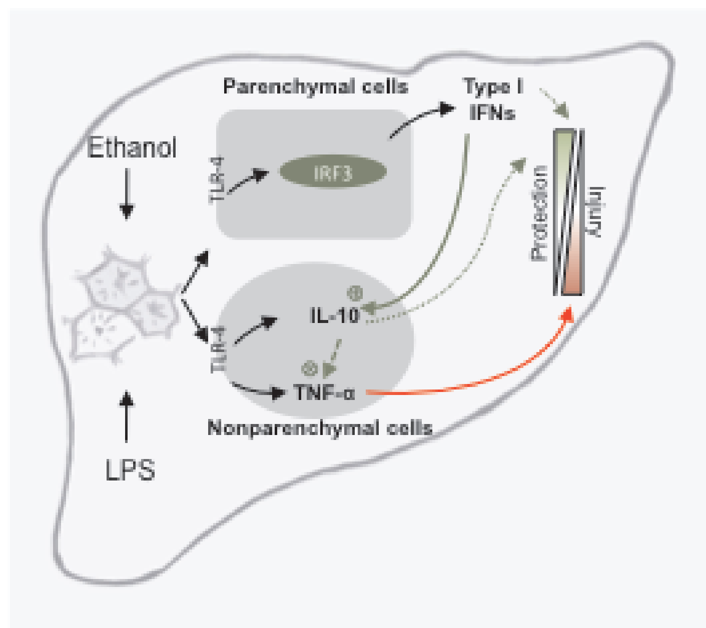

Figure 7.

VSL#3 Probiotic Treatment Attenuates Fibrosis Without Changes in Steatohepatitis in a Diet-Induced Nonalcoholic Steatohepatitis Model in Mice

Arumugam Velayudham, Angela Dolganiuc, Michael Ellis, Jan Petrasek, Karen Kodys, Pranoti Mandrekar, and Gyongyi Szabo

Nonalcoholic fatty liver disease (NAFLD) and its advanced stage, nonalcoholic steatohepatitis (NASH), are the most common causes of chronic liver disease in the United States. NASH features the metabolic syndrome, inflammation, and fibrosis. Probiotics exhibit immunoregulatory and anti-inflammatory activity. We tested the hypothesis that probiotic VSL#3 may ameliorate the methionine-choline-deficient (MCD) diet–induced mouse model of NASH. MCD diet resulted in NASH in C57BL/6 mice compared to methionine-choline-supplemented (MCS) diet feeding evidenced by liver steatosis, increased triglycerides, inflammatory cell accumulation, increased tumor necrosis factor α levels, and fibrosis. VSL#3 failed to prevent MCD-induced liver steatosis or inflammation. MCD diet, even in the presence of VSL#3, induced up-regulation of serum endotoxin and expression of the Toll-like receptor 4 signaling components, including CD14 and MD2, MyD88 adaptor, and nuclear factor κB activation. In contrast, VSL#3 treatment ameliorated MCD diet–induced liver fibrosis resulting in diminished accumulation of collagen and α-smooth muscle actin. We identified increased expression of liver peroxisome proliferator-activated receptors and decreased expression of procollagen and matrix metalloproteinases in mice fed MCD+VSL#3 compared to MCD diet alone. MCD diet triggered up-regulation of transforming growth factor beta (TGFβ), a known profibrotic agent. In the presence of VSL#3, the MCD diet–induced expression of TGFβ was maintained; however, the expression of Bambi, a TGFβ pseudoreceptor with negative regulatory function, was increased. In summary, our data indicate that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. The mechanisms of VSL#3-mediated protection from MCD diet–induced liver fibrosis likely include modulation of collagen expression and impaired TGFβ signaling. (HEPATOLOGY 2009;49:989-997.)

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease in the United States.[1,2] The advanced stage of NAFLD, nonalcoholic steatohepatitis (NASH), features liver inflammation and fibrosis, and has a strong association with the metabolic syndrome, including insulin resistance, dyslipidemia, and obesity.[1-4] The complexity and the chronology of pathophysiological events leading to development of NAFLD/NASH are not fully understood. Among mechanisms of inflammation, tumor necrosis factorα (TNFα) appears to play a critical role in both insulin resistance and hepatic inflammatory cell recruitment in NAFLD/NASH.[3-5] Furthermore, activation of nuclear factor κB (NF-κB), a master regulator of inflammation, has been demonstrated in nonalcoholic fatty livers.[3,6] Based on the similarities in the pathologic

Abbreviations: α-SMA, alpha-smooth muscle actin; Bambi, bone morphogenic protein and activin membrane-bound inhibitor; ip, intraperitoneal; LPS, lipopolysaccharide; MCD, methionine-choline-deficient; MCS, methionine-choline-supplemented; MMP, matrix metalloproteinase; MyD88, myeloid differentiation primary response gene 88; mRNA, messenger ribonucleic acid; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NF-κB, nuclear factor kappa B; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator 1α; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-response element; TLR, toll-like receptor; TNFα, tumor necrosis factor alpha; TGFβ, transforming growth factor beta; SC, stellate cells.

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changes in the liver in NASH and alcoholic steatohepatitis, it has been proposed that endotoxin from gut-derived gram-negative bacteria and lipopolysaccharide (LPS)-sensing machinery, including Toll-like receptor 4 (TLR4)/MyD88 pathways, may play a role in the pathogenesis of NASH.[7] Both fat metabolism and inflammation are regulated by peroxisome proliferator-activated receptors (PPARs),[1-3] suggesting that inflammation is a complex process regulated at different levels during development of NAFLD and its progression to NASH.

NASH can lead to liver fibrosis and cirrhosis.[1] Recent studies suggest that most "cryptogenic" cirrhosis is the result of previously undiagnosed NASH[8]; however, there are no known determinants of progressive liver damage at the cellular or molecular level and therapeutic modalities that could prevent progression of NASH are yet to be developed.

The human gut microflora is important in regulating host immune homeostasis.[9] *In vivo* administration of VSL#3, a probiotic preparation of live, freeze-dried bacteria containing eight bacterial species (*Streptococcus salivarius* subsp. *thermophilus*, *Bifidobacterium* [*B. breve*, *B. infantis*, *B. longum*], *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus*), attenuated liver steatosis in ob/ob mice that present characteristics of NAFLD.[10] Although the cellular and molecular basis of the action of probiotics is yet to be understood, some liver-related beneficial effects of VSL#3 treatment were found in a small cohort of patients with NAFLD.[11]

Based on the features of NAFLD/NASH in humans, which include steatosis and liver inflammation followed by fibrosis,[12] and based on the suggested anti-inflammatory properties of VSL#3 in animal models of chronic inflammation,[10,13] we entertained the hypothesis that probiotic treatment with VSL#3 will ameliorate MCD diet-induced NASH by modulation of liver inflammation and/or fibrosis.

Materials and Methods

Animals and Experimental Protocol. The study obeyed Institutional Animal Care and Use Committee regulations at the University of Massachusetts Medical School. Female C57BL/6 mice were fed a methionine-choline-deficient (MCD) diet or a methionine-choline-supplemented (MCS) diet; a group of MCD diet-fed mice also received VSL#3 (the protocol is detailed in Supporting Information). The MCD diet feeding represents an animal model of NAFLD/NASH, which reproduces several aspects of human diseases, such as liver steatosis, inflammation, and fibrosis.[14-16]

Preparation of serum and tissue, histopathological analysis, biochemical assays and cytokine detection were described previously[17,18] and are detailed in the Supporting Information.

Electrophoretic Gel Mobility Shift Assay. The electrophoretic gel mobility shift assay (EMSA) was performed using 5μg of nuclear protein; other proteins were quantified in western blot, as described previously,[17,18] and detailed in Supporting Information.

RNA Analysis. Total RNA was extracted from liver tissue, and messenger RNA (mRNA) analysis was performed using quantitative real-time polymerase chain reaction (qPCR) as described.[17,18] All specific mRNA levels were corrected for the 18S internal control results from the same sample. The specific PCR primer sequences used in this study are listed in Table 1.

Statistical Analysis. Statistical significance for *in vivo* tests was determined using nonparametric Kruskal-Wallis and Mann-Whitney tests. Data are presented as mean $\pm$ standard error; a P value ≤ 0.05 was employed as the statistical threshold of significance.

Results

VSL#3 Treatment Prevents Fibrosis but not Fatty Liver and Inflammation in the MCD-Diet–Induced Model of NASH. Administration of the MCD diet to C57BL/6 mice resulted in an a classical pathophysiological picture of NASH compared to MCS diet feeding: first, disturbed lipid metabolism was suggested by microvesicular and macrovesicular steatosis (Fig. 1A) and increased liver triglyceride levels (Fig. 1B); second, inflammation occurred as indicated by multiple foci of inflammatory cell accumulations in the livers (Fig. 1A) and by increased in serum (data not shown) and liver TNFα (Fig. 1C); and, third, liver fibrosis occurred, as indicated by increased accumulation of collagen (Fig. 1F) and α-smooth muscle actin (α-SMA; Fig. 1G). These changes induced

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Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Table 1. qPCR Primers

Target gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
18S	gta acc cgt tga acc cca tt	cca tcc aat cgg tag tag cg
TNFα	cac cac cat caa gga ctc aa	agg caa cct gac cac tct cc
TLR4	gcc ttt cag gga att aag ctc c	aga tca acc gat gga cgt gta a
CD-14	gga agc cag aga aca cca tc	cca gaa gca aca gca aca ag
MD-2	gac gct gct ttc tcc cat a	cat tgg ttc ccc tca gtc tt
MyD88	aga aca gac aga cta tcg gct	cgg cga cac ctt ttc tca at
PPARα	aac atc gag tgt cga ata tgt gg	agc cga ata gtt cgc cga aag
PPARγ	gga aga cca ctc gca ttc ctt	tcg cac ttt ggt att ctt gga g
PGC1α	aga cgg att gcc ctc att tga	tgt agc tga gct gag tgt tgg
TGF-β1	att cct ggc gtt acc ttg	ctg tat tcc gtc tcc ttg gtt
MMP-2	ttt gct cgg gcc tta aaa gta t	cca tca aac ggg tat cca tct c
MMP-9	tgc cca ttt cga cga cga c	gtc cag gcc gaa tag gag c
Procollagen-I	gct cct ctt agg ggc cac t	cca cgt ctc acc att ggg g
Bambi	aaa act tca gac ggg tgt gg	tgg tgc tgg aga aat cac ag

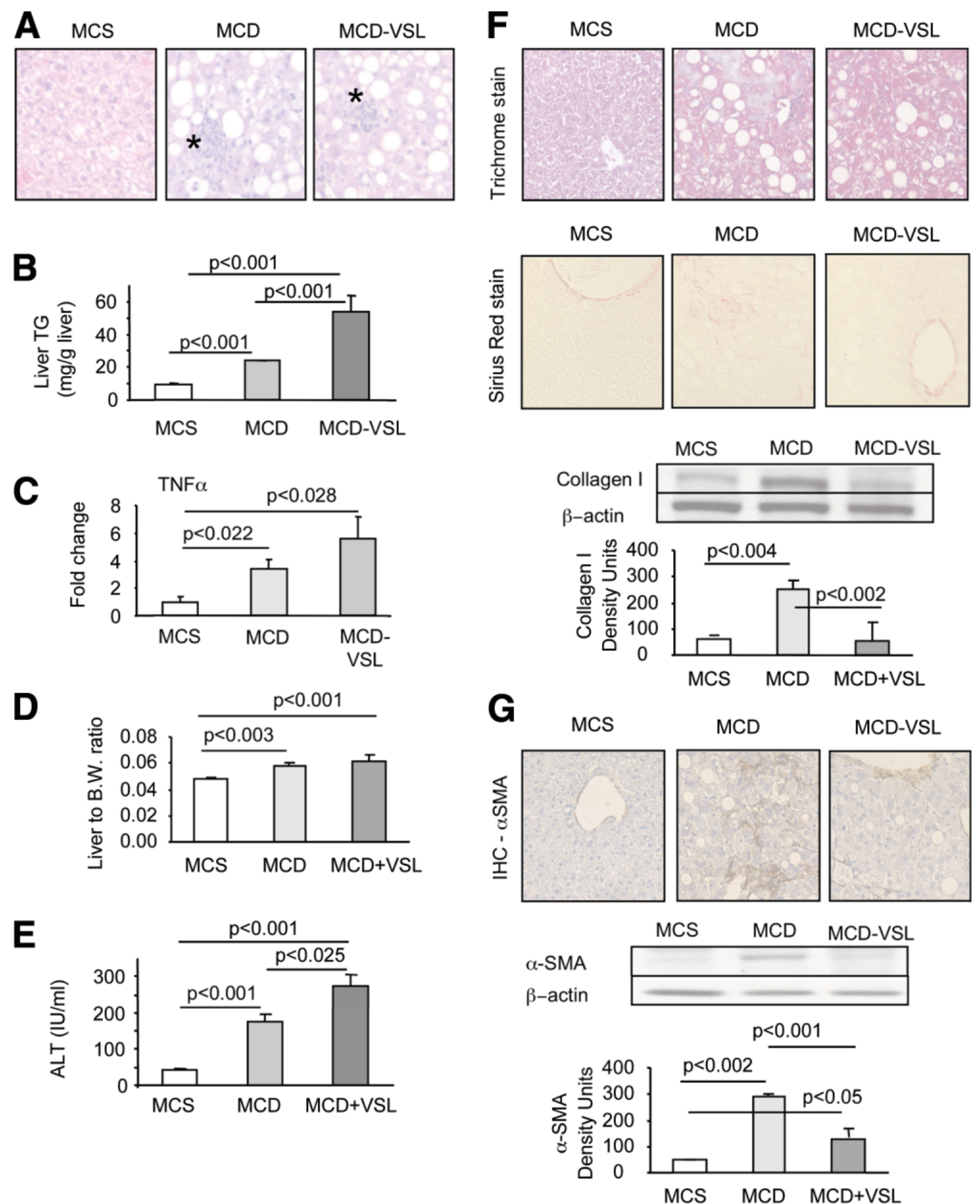

Fig. 1. VSL#3 treatment failed to prevent MCD diet–induced liver injury. Mice were fed a MCS or MCD diet for 10 weeks; VSL#3 was administered for the last 9 weeks of the MCD diet. Liver-to-body weight ratio was determined. Data are shown from six mice per experimental group. Liver histology was assessed after hematoxylin and eosin staining of liver tissue. Micrograph in (A) is a representative picture, with magnification 100×; the asterisk indicates inflammatory foci. (D) Liver/body ratio, (B) liver triglycerides (TG), (C) liver TNFα, and (E) serum ALT were determined from n = 6/group. (F) Liver sections were analyzed for collagen expression with trichrome (top panel) and Sirius Red (medium panel) staining; liver collagen I protein content was quantified in western blot using equal amounts of total liver proteins from each animal; one representative blot and densitometric analysis from n = 6/group (bottom panel) are shown. (G) Liver sections were analyzed for α-SMA expression using immunohistochemistry (top panel) staining; liver α-SMA protein content was quantified in western blot using equal amounts of total liver proteins from each animal; one representative blot and densitometric analysis from n = 6/group (bottom panel) are shown.

by the MCD diet feeding lead to increased liver-to-body weight ratio (Fig. 1D) and liver damage, as suggested by elevated serum alanine aminotransferase (ALT) level (Fig. 1E).

Coadministration of the probiotic VSL#3 had no significant effect on MCD diet–induced disturbance of fat metabolism because it failed to prevent steatosis (Fig. 1A) or to protect from accumulation of liver triglycerides (Fig. 1B). VSL#3 also failed to prevent the development of the inflammatory component of NASH as suggested by the lack of protection from inflammatory cell recruitment (Fig. 1A) or elevation of MCD diet+VSL#3-induced TNFα increase (Fig. 1C) compared to the MCD diet alone. VSL#3 treatment also failed to protect from MCD diet–induced liver injury (Fig. 1E,D).

In contrast, coadministration of VSL#3 reduced the progression of MCD diet–induced liver fibrosis as indicated by minimal evidence of collagen, identified by trichrome and sirius red staining (Fig. 1F) in MCD+VSL#3 diet–fed mice compared to mice fed the MCD diet alone. Further, the increased α-SMA expression in the livers of MCD diet–fed animals was significantly diminished by VSL#3 treatment as indicated by immunohistochemical staining (Fig. 1G). Consistent with the histology staining results, western blot analysis revealed increased collagen (Fig. 1F) and α-SMA (Fig. 1G) protein levels in the liver of mice subjected to MCD but not to MCS or MCD+VSL#3 diets. These results demonstrate that VLS#3 has a beneficial effect in the MCD-induced model of NASH. Our data also suggest that VSL#3 attenuated the fibrosis, but not the inflammation and liver damage, in MCD diet–induced steatohepatitis.

VSL#3 Treatment Inhibits Expression of Type I Collagen and Matrix Metalloproteinases. Collagen deposition and fibrosis are the result of stellate cell activation in the liver.[15,19] Recent evidence suggests that peroxisome proliferator-activated receptors (PPARs) may have antifibrotic effects.[20,21] PPARs, especially PPARα and PPARγ isoforms, not only play an important role in NAFLD through regulation of fat and glucose metabolism but also regulate stellate cell (SC) activity.[20,21] The effect of probiotics on PPAR activity is largely unknown. Based on our findings of antifibrotic effects of VSL#3, we hypothesized that VSL#3 treatment may modulate liver PPAR activity.

Investigation of liver mRNA levels revealed significantly increased PPARα (Fig. 2A) and PPARγ (Fig. 2B) levels in VSL#3-treated mice compared to MCD diet feeding alone. Although the PPAR activation and DNA binding were comparable in MCD-treated and MCD+VSL#3-treated mice (Fig. 2C), the antibody su-

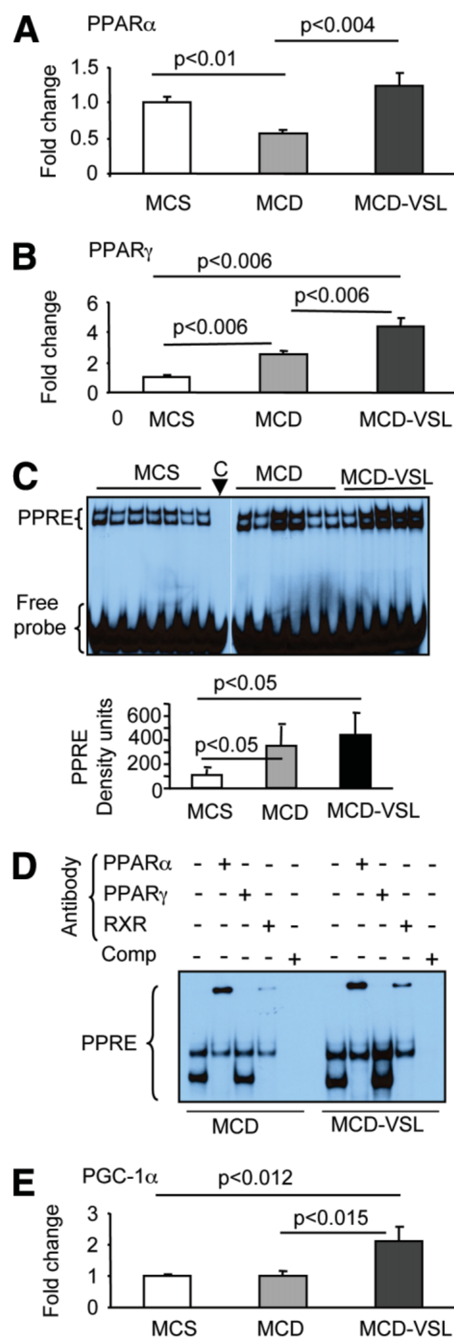

Fig. 2. VSL#3 induces expression of peroxisome proliferator-activated proteins. The expression of liver peroxisome proliferator-activated receptor-α (PPAR-α) (A), PPAR-γ (B), and PPAR-γ coactivator 1 α (PGC-1α (E) were assessed using qPCR. Data are shown as fold increase of MCD or MCD+VSL#3 group over control MCS diet with six mice pergroup. (C) Equal amounts of nuclear proteins were analyzed in EMSA for binding to the PPAR response element (PPRE). One sample was preincubated with cold PPRE oligonucleotide prior to EMSA as specificity control (C); a representative EMSA gel (top) and the densitometric analysis from six mice per group (bottom panel) are shown. (D) Equal amounts of nuclear proteins were analyzed preincubated with anti-PPARα, anti-PPARγ or anti-RXR antibodies and subjected to EMSA for binding to the PPRE; a cold competition control (Comp) was included as above. A representative EMSA gel (top) and the densitometric analysis from six mice per group (bottom panel) are shown.

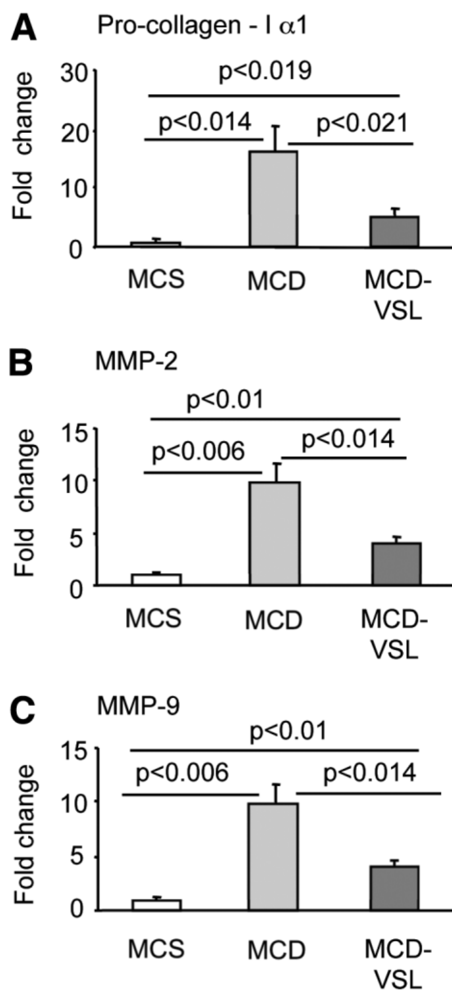

Fig. 3. VSL#3 limits the expression of MCD diet–induced matrix metalloproteinase in the liver. The liver RNA levels of (A) liver procollagen I-α1, (B) matrix metalloproteinase-2 (MMP-2), and (C) MMP-9, and the 18S control were analyzed using qPCR. Data are shown as fold increase of MCD or MCD+VSL#3 group over control MCS diet, all adjusted to 18S internal controls, with six mice per group.

pershift indicated a predominant presence of PPARα and retinoid X receptor (RXR), and to a lesser extent of PPARγ, in MCD-induced and MCD-VSL#3–induced PPAR activation (Fig. 2D). Downstream in the activation pathway, the mRNA levels of the PPAR-γ coactivator 1α (PGC-1α), a transcriptional coactivator of PPAR,[22] was significantly increased by VSL#3 treatment (Fig. 2E).

Administration of the MCD diet increased mRNA levels of procollagen 1α, and this response was attenuated in the presence of VSL#3 treatment (Fig. 3A). Changes in procollagen I-α1 mRNA levels closely mirrored changes in collagen protein levels and correlated with collagen expression detected by trichrome and Sirius red staining (Fig. 1F).

Changes in collagen expression also correlated with the expression of matrix metalloproteinases (MMPs) that play an important role in hepatic fibrosis.[23] SCs are important sources of MMP in the liver,[23,24] and PPAR activators modulate MMP expression.[25] Thus, we followed our finding of the elevated PPAR activity in the liver of MCD+VSL#3-fed compared to MCD-fed animals (Fig. 2) and predicted that first, the MCD diet will elevate liver MMP levels, and second, VSL#3 treatment will modulate liver MMP levels. Indeed, we found a significant induction of MMPs, including MMP-2 and MMP-9, in livers of animals on the MCD diet (Fig. 3B,C). Confirming our hypothesis, coadministration of VSL#3 significantly attenuated the MCD diet–induced liver expression of both MMP-2 and MMP-9 (Fig. 3B,C).

Diet-Induced Steatohepatitis Is Associated with Increased Serum Endotoxin Levels, Increased Expression of the TLR4 Receptor Complex, and Hyperresponsiveness to LPS Stimulation. Previous studies suggested that gut-derived endotoxin plays a key role in development and progression of NASH.[1-3,5-7] Endotoxin is a potent activator of liver parenchymal and nonparenchymal cells, of which Kupffer cells and SCs govern the development of NASH.[26] Further, probiotic bacteria prevent hepatic damage and maintain colonic barrier function in a mouse model of sepsis.[13] We identified a moderate but statistically significant increase in serum LPS levels in mice with MCD diet–induced steatohepatitis compared to MCS control diet–fed mice (Fig. 4). However, VSL#3 treatment did not significantly affect serum endotoxin levels compared to the MCD diet alone (Fig. 4). These data were in agreement with our findings that elevated serum (data not shown) and liver TNFα (Fig. 1C) and ALT (Fig. 1E) levels were not affected by VSL#3 treatment, because endotoxin is a major stimulator of the TNFα-producing immune cells, and TNFα plays a key role in liver damage during NASH.[26,27]

Based on these findings, we speculated that VSL#3 treatment would fail to prevent the activation of endotoxin-triggered inflammatory activation in the MCD diet–

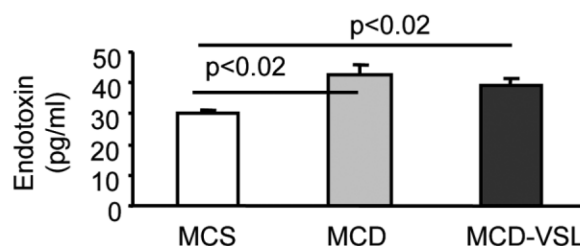

Fig. 4. VSL#3 failed to protect from MCD diet–induced endotoxemia. Mice were fed MCS or MCD diet for 10 weeks; VSL#3 was administered for the last 9 weeks of the MCD diet. Serum levels of endotoxin were analyzed at the end of the 10-week feeding period using a Limulus Amebocyte Lysates assay. Mean ± standard error data from six mice per group are shown.

induced NASH model. LPS induces activation of the proinflammatory cascade and cellular activation via the TLR4 complex expressed on most liver cells, including nonparenchymal (Kupffer cells and SCs), immune inflammatory cells, and hepatocytes.[28] Thus, increased expression of the components of the TLR4 receptor complex, including TLR4, its coreceptors CD14 and MD-2, and the common TLR adapter MyD88 may increase cellular responses to LPS.[28,29] We found that RNA levels of CD14 (Fig. 5A), MD-2 (Fig. 5B), and MyD88 (Fig. 5C) were significantly up-regulated after MCD or MCD+VSL#3 diet feeding compared to the control MCS diet. Further, MD-2 (Fig. 5B) mRNA levels were increased by VSL#3 treatment whereas there was no significant change in the mRNA levels of TLR4 (Fig. 5E) between mice on the different diets. These results suggested that MCD diet–induced up-regulation of the TLR4 coreceptors CD14 and MD-2, and molecules involved in TLR4 downstream signaling, such as MyD88, may sensitize livers with steatohepatitis to increased responsiveness to LPS.

Activation of TLR4 triggers downstream signaling that culminates in activation of nuclear transcription factors.[28,29] Among those, the NF-κB pathway plays a key role in activation of Kupffer cells and SCs during liver diseases.[26] We found that baseline activation of NF-κB was statistically similar in all analyzed groups (Fig. 5E). Based on the fact that NASH is a multihit disease,[1-3,5-7] we further employed an exogenous LPS administration strategy to reveal the physiological relevance of our above-described findings in the LPS-sensing receptor complex. LPS challenge resulted in significantly higher NF-κB nuclear translocation and DNA binding in the livers of mice fed the MCD diet compared to mice fed the MCS control diet (Fig. 5E). Furthermore, VSL#3 administration augmented the LPS-induced NF-κB activation in MCD diet–fed mice (Fig. 5E). These results suggested that MCD diet–induced steatohepatitis activated proinflammatory cytokine induction pathways and VSL#3 treatment failed to attenuate the exaggerated proinflammatory activation in response to LPS.

VSL#3 Modulates Transforming Growth Factor-β Signaling Pathways. Our data suggested so far that VSL#3 failed to ameliorate inflammation, yet prevented

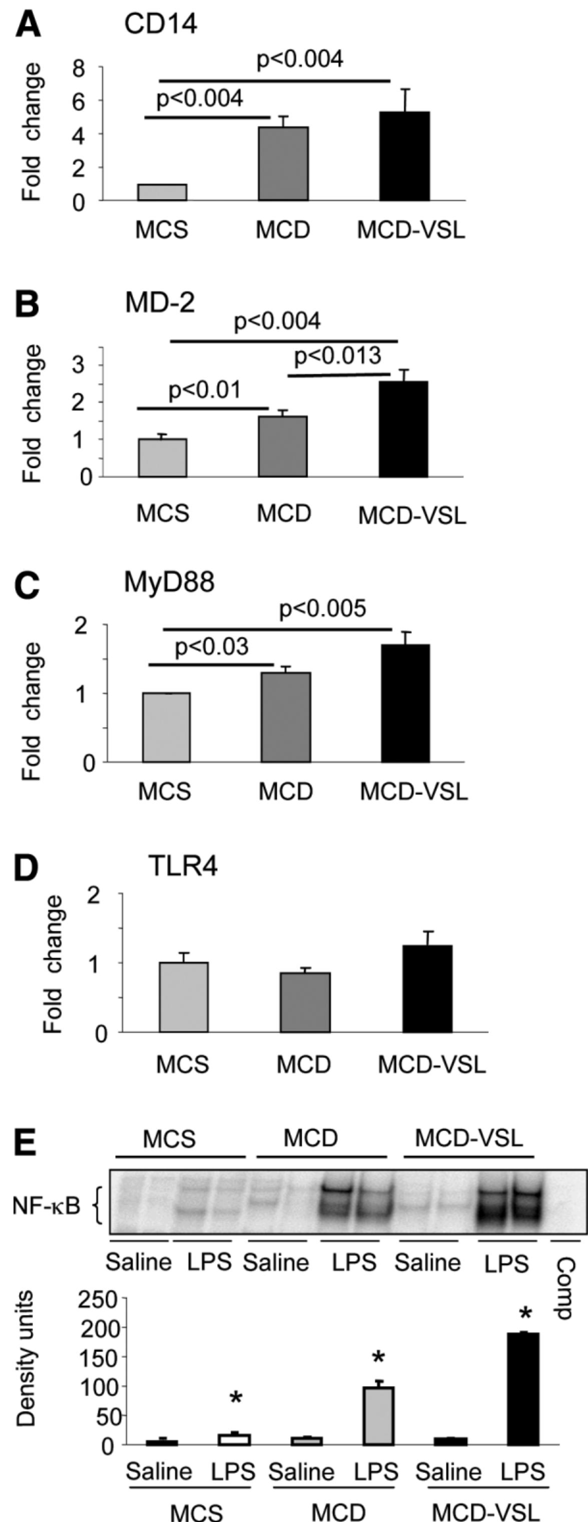

Fig. 5. VSL#3 augments MCD diet–induced modulation of the LPS signaling complex. The liver mRNA levels of (A) CD14, (B) MD-2, (C) MyD88, (D) toll-like receptor (TLR) 4, and 18S were analyzed using qPCR. Data are shown as fold increase of MCD or MCD+VSL#3 group over control MCS diet, all adjusted to corresponding 18S housekeeping controls, with six mice per group. (E) Mice were fed MCS or MCD diet for 10 weeks; VSL#3 was administered for the last 9 weeks of the MCD diet. At the end of the 10-week feeding period, the animals were challenged with LPS (0.5 mg/kg body weight, i.p. for 1.5 hours). Liver nuclear extracts were analyzed for NF-κB binding activity in EMSA using specific radioisotope-labeled oligonucleotides; 20× excess of unlabeled oligonucleotide was used for cold competition (Comp). A representative gel is shown on the top and the densitometric analysis from six mice per group is shown on the bottom of each panel. Asterisk represents $P < 0.01$ compared to the saline group with the same diet feeding.

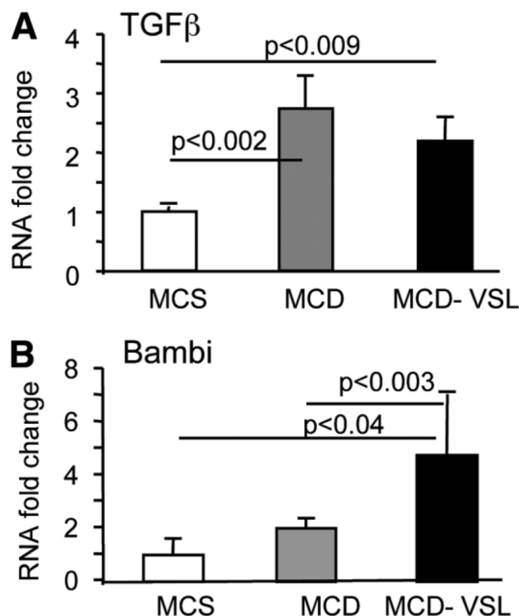

Fig. 6. VSL#3 modulates TGFβ pathway. The liver RNA levels of (A) TGFβ, (B) Bambi, and 18S were analyzed using qPCR. Data are shown as fold increase of MCD or MCD+VSL#3 group over control MCS diet, all adjusted to corresponding 18S housekeeping controls, with six mice per group.

fibrosis in the MCD diet–induced model of NASH. Although inflammation has been suggested as a prerequisite for development of fibrosis,[7,12,26] the chronology and interdependence of inflammation and fibrosis are yet to be fully understood in NASH. More recently, a link between proinflammatory and profibrogenic signals was suggested.[26] Seki et al. indicated that during liver inflammation, LPS down-regulates the transforming growth factor-beta (TGFβ) pseudoreceptor, Bambi, to sensitize SCs to TGFβ-induced signals from inflammatory cells in a TLR4/MyD88-NF-κB–dependent manner, thus modulating liver fibrosis.[26] Based on our data, we predicted that the MCD diet–induced NASH could modulate TGFβ expression and/or signaling due to endotoxemia and LPS-receptor–mediated signaling, similar to changes seen in other inflammation models.[26] We further hypothesized that VSL#3 treatment could modulate the MCD diet–induced changes in expression of Bambi, the TGFβ pseudoreceptor, and thus disrupt the profibrotic TGFβ signaling pathway, despite ongoing inflammation. TGFβ RNA levels were increased by the MCD diet (Fig. 6A), suggesting that the MCD-triggered TGFβ could contribute to SC activation and collagen production (Figs. 1 and 3). In the presence of VSL#3 during the MCD diet, TGFβ levels showed a decreasing trend that was not statistically significant (Fig. 6A). However, we found that Bambi was significantly up-regulated in the presence of VSL#3 treatment compared to MCD diet alone (Fig. 6B). These data suggested that VSL#3 treatment promoted the expression of the TGFβ pseudoreceptor Bambi that could arrest the SCs in a quiescent state.

Discussion

Our study shows that VSL#3 treatment prevents fibrosis in the MCD diet–induced NASH model without significant attenuation of the ongoing steatohepatitis. This observation supports the concept that *in vivo* fibrosis and steatohepatitis can be regulated independently[30,31] and points to a potentially new therapeutic application of VSL#3.

The current view on the mechanisms and progression of NASH favors a model in which steatosis and then steatohepatitis are induced as a result of fatty acid overload and inflammation, leading to subsequent activation of SCs that produce collagen and lead to liver fibrosis.[1-3,5-7,26] The key component in the mechanisms of fibrosis in the liver is the activation of SCs that are the primary source of α-SMA and collagen deposition.[14,20,26] SC activation is induced by multiple insults, including TNFα, and TGFβ.[4,5,7,26] We identified increased TNFα production in MCD diet–induced NASH, which remained elevated in MCD+VSL#3-treated mice, in agreement with studies from Ewaschuk et al.[13] and Hart et al.[32] TNFα modulates SC activation via a mechanism that involves inhibition of PPAR expression and its binding to the peroxisomal proliferator response element (PPRE).[33] We found an increase in PPAR mRNA levels in the livers of MCD+VSL#3-treated mice compared to those treated with the MCD diet alone. Although there were no significant differences in the levels of PPAR activity or in composition of the PPAR complex, we identified that VSL#3 treatment during MCD diet led to an increase in PGC-1a and a decrease in Col1a, which are targets of PPARs.[34,35] Taken together, these changes suggested a role for PPARs in the antifibrotic effects of VSL#3. However, we identified that MCD diet up-regulated TGFβ, a known SC activator.[26] Because TGFβ regulates collagen production,[30,36] increased TGFβ production could contribute to the MCD-induced fibrosis. VSL#3 inhibited fibrosis and, importantly, triggered the production of Bambi, a transmembrane protein highly similar to TGFβ receptors.[37] In contrast to regular TGFβ receptor, the intracellular domain of Bambi is short and lacks a serine/threonine kinase domain that is essential for transducing TGFβ signals; thus, Bambi functions as a pseudoreceptor and acts as a negative regulator of TGFβ signaling pathway.[26,37] To date, the fine mechanisms of Bambi regulation are not fully understood. However, several authors reported that the bone morphogenic protein family, which also includes Bambi,[37] is regulated via NF-κB–dependent

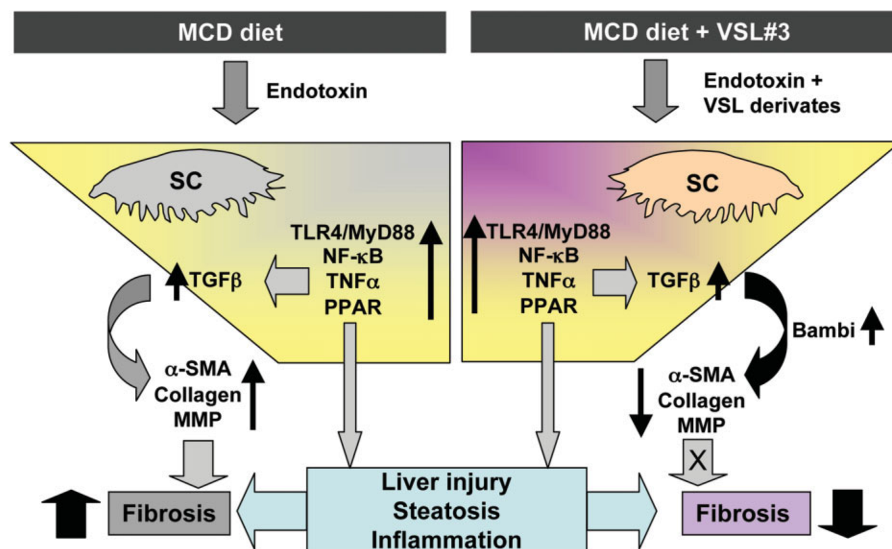

Fig. 7. Hypothetical model of the effects of VSL#3 on MCD-induced NASH model.

mechanisms.[26,38-40] We report increased NF-κB activity and elevated expression of Bambi in MCD diet–fed VSL#3-treated group compared to controls fed the MCD diet alone. Further, Bambi RNA changes mirror the protein levels, and Bambi expression is restricted to SCs of the liver.[26] Thus, in the presence of VSL#3, high levels of Bambi could prevent TGFβ-induced signals, and control the unrestricted activation of SCs by ongoing inflammation. These data are in agreement with those of Seki et al., who showed that down-regulation of Bambi mRNA and protein expression, and subsequent sensitization to TGFβ signals, is mediated by a MyD88/NF-κB–dependent pathway and occurs with ongoing liver inflammation.[26]

We identified increased expression of the components of the signaling pathway initiated by LPS via TLR4, including CD14, MyD88, and NF-κB, during MCD-induced NASH and these were further exacerbated in MCD+VSL#3-treated mice. Thus, the increased serum LPS levels in the MCD diet–fed mice are likely to contribute to the sustained inflammation. In light of these findings, and taking into consideration the significantly higher levels of serum ALT in VSL#3-exposed animals compared to MCD diet–fed controls, it is possible that VSL#3 treatment not only failed to inhibit but also augmented MDC diet–induced inflammation; such a conclusion could not be reached because of the imprecise nature of histological scoring. VSL#3 has TLR2-stimulating and TLR9-stimulating capacity[41]; both TLR2 and TLR9 share the MyD88-dependent signaling pathway with TLR4.[28,29] We did not identify changes of TLR2 levels (data not shown); however, the increased expression of MyD88 in VSL#3-exposed animals could accommodate signaling via TLR2, TLR4, or TLR9. We also acknowledge that the presence/processing of VSL#3 *in vivo* is needed in order to achieve antifibrotic effects; in this context, the effects of VSL#3 on gut, the gut microbiota-liver relationship, the detailed composition of VSL#3-derived microbial products, and their specific interactions with stellate cells at biochemical and mechanistic levels remain the subject of future research.

In summary, our data indicate that VSL#3 modulates liver fibrosis but does not protect from inflammation and steatosis in NASH. Within the limitations of the animal model,[14,15,18,42] our current working model takes into consideration a role for the endotoxin/TLR4/MyD88 pathway, but also acknowledges the differential contributions of TNFα-mediated, NF-κB–mediated, PPAR-mediated, and TGFβ/Bambi-mediated activation pathways toward development of inflammation and fibrosis during NASH (Fig. 7). Our results suggest that, at least in the NAFLD/NASH model, the benefit of the VSL#3 treatment on fibrosis may occur even in the absence of significant changes in markers of inflammation and fat in the liver.

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Type I Interferons Protect from Toll-like Receptor 9-Associated Liver Injury and Regulate IL-1 Receptor Antagonist in Mice

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Type I Interferons Protect from Toll-like Receptor 9-Associated Liver Injury and Regulate IL-1 Receptor Antagonist in Mice

Short title: Type I interferons in TLR9-associated liver injury

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Department of Medicine, University of Massachusetts Medical School

Jan Petrasek - Acquisition of data, analysis and interpretation of data, statistical analysis and manuscript writing
Angela Dolganiuc - Acquisition of data, analysis and interpretation of data, and manuscript writing
Evelyn A. Kurt-Jones - Intellectual content, critical revision of the manuscript
Timea Csak - Acquisition of data, analysis and interpretation of data
Gyongyi Szabo - Study concept and design, critical revision of the manuscript, and obtained funding

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Abbreviations: CpG, cytidine-phosphate-guanosine-rich DNA; IFN, interferon; IFNAR1, type I interferon receptor; IL-1ra, interleukin 1 receptor antagonist; IL-1β, interleukin 1 beta ; IRF, interferon regulatory factor; ISG, interferon stimulated gene; LPS, lipopolysaccharide; LTA, lipoteichoic acid; NFκB, nuclear factor κB; TLR, toll-like receptor

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Disclosures: nothing to disclose

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Abstract:

Background & Aims: Liver inflammation and injury are mediated by the innate immune response, which is regulated by Toll-like receptors (TLR). Activation of TLR9 induces Type I interferons (IFNs) via the interferon regulatory factor (IRF)-7. We investigated the roles of Type I IFNs in TLR9-associated liver injury in mice.

Methods: Liver injury was induced in wild-type (WT), IRF7-deficient, and IFN-α/ß receptor-1 (IFNAR1)-deficient mice by administration of ligands for TLR9 or TLR2. Findings from mice were verified in cultured hepatocytes and liver mononuclear cells, and *in vivo* experiments using recombinant Type-I IFN and interleukin-1 receptor antagonist (IL-1ra).

Results: Type I IFNs were upregulated during TLR9-associated liver injury in WT mice. IRF7- and IFNAR1-deficient mice, which have disruptions in Type I IFN production or signaling, respectively, had greater amounts of liver damage and inflammation, decreased recruitment of dendritic cells, and increased production of TNF-α by liver mononuclear cells (LMNC). These findings indicate that Type I IFNs have anti-inflammatory activities in liver. The IL-1ra, which is produced by LMNC and hepatocytes, is an IFN-regulated antagonist of the pro-inflammatory cytokine IL-1β; IRF7- and IFNAR1-deficient mice had decreased levels of IL-1ra, compared with WT mice. IL-1ra protected cultured hepatocytes from IL-1β-mediated sensitization to cytotoxicity from TNF-α. In vivo exposure to Type I IFN, which induced IL-1ra, or administration of IL-1ra reduced TLR9-associated liver injury; the protective effect of Type-I IFNs therefore appears to be mediated by IFN-dependent induction of IL-1ra.

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Conclusions: Type I IFNs have anti-inflammatory effects mediated by endogenous IL-1ra which regulates the extent of TLR9-induced liver damage. Type I interferon signaling is therefore required for protection from immune-mediated liver injury.

KEY WORDS: liver disease; immunology; innate immunity; bacterial DNA

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Liver-related etiologies are among the top ten leading causes of death in the United States.[1] The common hallmark of most liver diseases is inflammation and injury, which may result in acute liver failure, or, if persist, promote the development of fibrosis and eventually cirrhosis.[2] Presently, liver inflammation and injury have no pathogenesis-specific treatment, but they share a common pathway of induction of innate immune responses, triggered by Toll-like receptors (TLRs).[3]

For example, TLR9 is activated by unmethylated DNA rich in cytidine-phosphate-guanosine (CpG). This motif is present in bacterial DNA and also in DNA from apoptotic mammalian cells.[4] Emerging data provide evidence for the role of CpG DNA and TLR9-mediated inflammation in acute and chronic liver injury of diverse origin, including alcoholic liver disease,[5] primary biliary cirrhosis,[6] primary sclerosing cholangitis[7] and acetaminophen-induced liver injury.[8] TLR9-initiated signals are also involved in general processes such as liver fibrosis,[9, 10] liver cirrhosis,[11] ischemia-reperfusion injury,[12] and liver graft rejection[13]. TLR9 acts synergistically with the TLR2 ligand lipoteichoic acid (LTA),[14] and further sensitizes the liver to injury induced by the TLR4 ligand lipopolysaccharide (LPS).[15, 16]

Activation of TLR9 by CpG results in increased production of inflammatory mediators via the nuclear factor κB (NFκB), and in a strong induction of Type I interferons (IFN-α and IFN-β) via the interferon regulatory factor 7 (IRF-7),[17] which together with IRF3 also plays a role in TLR4-dependent induction of Type I IFNs. [18] While NFκB-dependent induction of inflammatory cytokines by CpG is considered the key event in the TLR9-mediated liver injury,[5, 6, 8, 13] little is known about the role of IRF7-dependent Type-I IFN induction and signaling after TLR9 stimulation.

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Here we demonstrate that induction of Type I IFNs and Type I IFN-mediated signaling has a protective role in TLR9-induced liver inflammation and injury. We report that impaired induction of interferon-stimulated genes, including the anti-inflammatory cytokine, interleukin 1 receptor antagonist (IL-1ra), increases liver inflammation and injury. Our results also demonstrate a protective effect of the IL-1ra *in vivo* in TLR9-associated liver injury.

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Experimental procedures

Animals and experimental protocol

The B6.129F2 and C57Bl/6 wild-type (WT) mice were purchased from Jackson Laboratory. IRF7-deficient (IRF7-/-) mice on B6.129F2 background were provided by Tadagatsu Tanaguichi (Tokyo) and type I interferon α/β receptor 1-deficient (IFNAR1-/-) mice on the C57Bl/6 background were the kind gift of Jonathan Sprent (Scripps Research Institute, La Jolla, CA). All animals (3-6/experimental group) were 6–8 weeks old and received proper care in agreement with animal protocols approved by the Institutional Animal Use and Care Committee of the University of Massachusetts Medical School.

We employed a previously described model of TLR9-associated liver injury induced by administration of TLR9 and TLR2 ligands.[15, 16] After acclimatization, WT, IRF7-/- and IFNAR1-/- mice were injected intraperitoneally (i.p.) with saline or the combination of 2.5 mg/kg unmethylated DNA rich in cytidine-phosphate-guanosine (CpG, ODN1826 murine TLR9 ligand; InvivoGen, San Diego, CA), and 5 mg/kg lipoteichoic acid (LTA, from Staphylococcus aureus; Sigma, Saint Louis, MO). Three days after the above priming stimulus, the mice were injected i.p. with either saline or 0.5 mg/kg lipopolysaccharide (LPS, from Escherichia coli 0111:B4, Sigma, St. Louis, MO) and sacrificed as indicated. Some C57Bl/6 WT mice received a single i.p. injection of 100,000 IU human pegylated interferon alpha-2b (pegIFNα2, Pegintron, Schering, Kenilworth, NJ) two hours prior to LPS. Others were pretreated with recombinant human interleukin-1 receptor antagonist (IL-1ra) 25 mg/kg i.p. every six hours (Anakinra, Amgen, Thousand Oaks, CA) for 24 hours before CpG+LTA, and the treatment with IL-1ra was ongoing until sacrifice. Serum was separated by centrifugation. Livers were snap frozen, stored in

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RNAlater (Qiagen GmbH, Hilden, Germany) or fixed in 10% neutral-buffered formalin. ALT was quantified by biochemical assay (D-Tek Analytical Laboratories Inc, San Diego, CA).

Histopathology analysis

Sections of formalin-fixed, paraffin-embedded livers were stained with hematoxylin and eosin (H&E), and assessed for inflammatory infiltrate; area of inflammatory infiltrates was calculated with Microsuite (Olympus Soft Imaging Solution GmbH, Munster, Germany) image analysis software in 20 high power fields.

Isolation of hepatocytes and liver mononuclear cells

Animals received anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg); the livers were perfused with saline solution followed by *in vivo* digestion, as we previously described.[19] The hepatocytes and liver mononuclear cells (LMNCs) were purified by centrifugation at slow speed (500g) and in Percoll gradient, respectively.

Phenotype analysis by flow cytometry

Cells were washed in PBS and incubated with anti-CD68 (FITC), anti-CD11c (FITC) or anti-PDCA1 (Alexa Fluor 647) antibodies for 30 minutes on ice. After incubation, cells were washed with PBS, fixed in paraformaldehyde and analyzed by flow cytometry. All antibodies were from eBioscience (eBioscience, Inc., San Diego, CA).

In vitro cell culture

Primary hepatocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1% insulin, transferrin, and selenium supplement on collagen-

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coated plates (Becton Dickinson Labware, Bedford, MA). Primary LMNCs were cultured in DMEM with 10% FBS. Hepatocytes were treated with recombinant murine IFN-α2b (500 IU/mL, eBioscience, San Diego, CA), murine IL-1ra (100 pg/mL, R&D Systems, Minneapolis, MN), murine IL-1β (100 IU/mL, Peprotech Inc., Rocky Hill, NJ) or murine TNF-α (0-100 ng/mL, Peprotech Inc., Rocky Hill, NJ). LMNCs were treated with mouse IFN-α2b or LPS (100 ng/mL, Sigma, St. Louis, MO).

Hepatocyte cytotoxicity assay

Lactate dehydrogenase (LDH) release from the hepatocyte into the culture supernatants was measured using the LDH-cytotoxicity assay kit (Abcam, Cambridge, MA), and normalized to total LDH (determined after treatment of cells with detergent-based lysis solution).

Cytokine and chemokine measurement

Serum concentrations of the secreted forms of interleukin-1β (IL-1β) and IL-1 receptor antagonist (IL-1ra) were analyzed with ELISA (R&D Systems, Minneapolis, MN). Monocyte chemotactic protein 1 (MCP-1) in the liver lysate was measured with ELISA from R&D, and TNF-α in cell culture supernatants was analysed using ELISA from BD Biosciences (BD Biosciences, San Jose, CA).

RNA analysis

RNA was extracted from liver tissue using the RNeasy kit (Qiagen Sciences, Maryland, USA) and on-column DNase digestion was performed using the DNase Set (Qiagen GmbH, Hilden, Germany); cDNA was synthesized with Reverse Transcription System (Promega Corp., Madison, WI). Real-time quantitative polymerase chain reaction (qPCR) was performed using

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the iCycler iQ Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) and specific primers (Supplementary Table 1).

Statistical Analysis

The data are presented as mean ± SEM. Comparison of the means was performed using Student's T-test or Kruskall-Wallis test, when appropriate. P values less than 0.05 were considered significant.

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Results

Type I IFNs are induced in TLR9-associated liver injury

TLR9 signaling induces Type I IFNs, which may play a role in tissue injury and regeneration. [20, 21] We have previously demonstrated that MyD88, a common adaptor to TLR9, TLR2 and TLR4 is critical in sensitization to liver injury.[16] Here we employed a model of TLR9-associated liver injury owed to sensitization by the synergistic effect of the TLR9 ligand CpG and the TLR2 ligand LTA, followed by a second hit with LPS,[15, 16] and asked if the integrity of the Type I IFN pathway was involved in liver damage. We found that combined administration of TLR9 and TLR2 ligands induced and sensitized the liver to injury by the TLR4 ligand LPS, as indicated by increased serum ALT levels in WT mice (Fig. 1A). More importantly, TLR9+TLR2 ligands induced expression of IFNα (Fig. 1B) and IFNβ (Fig. 1C) mRNA in the liver and expression of both IFNα and IFNβ was further upregulated upon TLR4 stimulation in WT mice. We also found that expression of the Type I IFN-inducible gene, interferon regulated gene ISG15, was induced by TLR9+TLR2 ligand treatment and the expression was further increased upon subsequent LPS stimulation (Fig. 1D). Collectively, these data suggested that Type I IFNs and IFN-triggered signaling pathways were upregulated in TLR9-associated liver injury.

Deficiency in Type I IFN induction exacerbates liver injury

Our data indicated activation of Type I IFN signaling in TLR9-associated liver injury. While both TLR9 and TLR2 ligands activate production of proinflammatory cytokines, TLR9 stimulation also triggers Type I IFNs.[17] Type I IFN production induced by TLR9 is largely

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dependent on activation of intracellular pathways involving interferon regulatory factor-7 (IRF7).[22] To define the importance of Type I IFN in TLR9-associated liver injury, we tested IRF7-deficient mice.

In sharp contrast to their wild-type littermates, IRF7-deficient mice showed exacerbation of TLR9-associated liver injury, as indicated by serum ALT elevations (Fig. 2A). Histopathology analysis (Fig. 2B) of IRF7-deficient livers revealed an about 5-fold increase in the number of inflammatory infiltrates (10.33 ± 3.71 in IRF7-KO vs. 1.75 ± 0.48 in WT, $P = 0.026$) and an about 2.5-fold increase in total area of inflammatory infiltrates (0.67 ± 0.05 vs. 0.28 ± 0.05 mm^2, $P = 0.017$), compared to wild-type controls.

The increased TLR9-associated liver injury in IRF7-deficient mice was accompanied by deficient induction of IFNα (Fig. 2C) and IFNβ (Fig. 2D), compared to controls. LPS stimulation could partially overcome the deficit in IFNα and IFNβ expression in TLR9+TLR2 primed mice, presumably by the direct effect of TLR4 signaling on Type I IFN induction.[23] However, there was decreased induction of the IFN-inducible genes, ISG15 (Fig. 2E) and IP-10 (Fig. 2F), in IRF7-deficient mice and further expression of these molecules upon stimulation with LPS was limited. These data supported our hypothesis that Type I IFN induction plays a role in the pathogenesis of TLR9-induced liver damage and suggested a protective role for Type I IFNs in liver injury.

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Deficiency in Type I IFN signaling exacerbates liver injury

The effects of Type I IFNs are mediated by a cognate receptor composed of two chains, IFNAR1 and IFNAR2, both of which are essential to initiate production of the interferon-stimulated genes (ISGs).[24] To differentiate between the direct and indirect protective effects of Type-I IFNs in TLR9-associated liver injury, we tested mice deficient in type I interferon receptor expression (IFNAR1-deficient mice).

Similar to findings in IRF7-deficient mice, mice deficient in IFNAR1 expression showed significantly increased serum ALT (Fig. 3A), widespread liver inflammation, about 5-fold increase in the number of inflammatory foci (4.80 ± 0.49 in IFNAR1-KO vs. 1.0 ± 0.58 in WT, $P = 0.018$) and about 7-fold increase in total area of inflammatory infiltrate (0.40 ± 0.11 vs. 0.06 ± 0.04 mm^2, $P = 0.036$) after TLR9+TLR2 stimulation compared to wild-type controls (Fig. 3B).

Type I IFNs *per se* are IFN-sensitive and are strictly regulated by a self-initiated amplification loop involving IRF7 and both IFN receptor chains.[24] Consistent with this mechanism, IFNAR1-deficient mice showed substantially lower induction of liver IFNα (Fig. 3C), IFNβ (Fig. 3D) and minimal expression of ISG15 (Fig. 3E) and IP-10 (Fig. 3F), compared to controls. This difference was not overcome by LPS stimulation. Collectively, these data suggested that both Type I IFN induction and IFN-induced signaling may play a protective role in the liver by limiting inflammatory infiltrate and liver injury.

Deficiency of Type I IFN signaling results in decreased dendritic cell recruitment to the liver in TLR9-associated injury

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The exaggerated liver inflammatory infiltrate in IRF7- and IFNAR1-deficient mice suggested a role for Type I IFNs in recruitment of inflammatory cells in TLR9-associated liver injury. We thus aimed to define the cell composition of these inflammatory infiltrates. Flow cytometric analysis of liver mononuclear cells (LMNCs) showed equal enrichment of CD68$^+$ monocytes/macrophages in the livers of both WT and IFNAR1-deficient mice after TLR9+TLR2 priming (Fig. 4A). Similarly, liver expression of chemokines MCP-1, MCP-2, MIP-1α and MIP-1β (Suppl. Fig. 1A-E) and chemokine receptors CCR1, CCR2 and CCR5 (Suppl. Fig. 1F-H), which are involved in macrophage recruitment, was upregulated in TLR9-associated liver injury to a comparable extent in both WT and IFNAR1-deficient mice.

In contrast to WT mice, LMNCs isolated from mice deficient in IFNAR1 showed a significantly lower proportion of CD11c$^+$ (myeloid) and PDCA1$^+$ (plasmacytoid) dendritic cells (DC) (Fig. 4B). This finding was associated with a significantly decreased liver expression of the chemokine ligand 21 (CCL-21) (Fig. 4C), a key molecule involved in recruitment of DC, [25] and decreased expression of the CCL-21 receptor CCR7 (Fig. 4D). These data support our hypothesis that in TLR9-associated liver injury, Type I IFNs are required for dendritic cell recruitment to the liver.

Deficient Type I interferon signaling results in an imbalance in IL-1β / IL-1 receptor antagonist induction in TLR9-associated liver injury

The protective effect of Type I IFN induction and signaling against TLR9-associated liver injury implied the involvement of Type I IFN-dependent anti-inflammatory factors. We thus analyzed

the expression of another IFN-dependent gene, IL-1 receptor antagonist (IL-1ra), which is a natural endogenous antagonist of the proinflammatory interleukin-1β (IL-1β) at the receptor level.[26] Pro-IL-1β gene in the liver (Fig. 5A) and IL-1β protein (Fig. 5B) in the serum were significantly induced by TLR9+TLR2 ligands and further upregulated by LPS to a comparable extent in WT, IRF7- and IFNAR-deficient mice (Fig. 5A,B). Induction of IL-1ra mRNA in the liver and of the secreted IL-1ra protein in serum were decreased both in IRF7- and IFNAR1-deficient mice treated with CpG+LTA, compared to controls (Fig. 5C,D). More important, sensitization via TLR9+TLR2 ligands resulted in less IL-1ra protein production upon stimulation with LPS in both IRF7- and IFNAR1-deficient mice compared to their wild-type controls (Fig. 5D). This finding suggested that aggravated TLR9-associated liver injury in Type I IFN deficient animals is associated with a significant imbalance in IL-1β/IL-1ra signaling.

IL-1ra protects hepatocytes from IL-1β-dependent sensitization to cell death induced by TNF-α

Our data suggested a protective effect of IL-1ra in TLR9-associated liver injury. To gain a mechanistic insight, we first investigated the source of IL-1ra in the liver. We observed that WT primary hepatocytes and LMNCs stimulated with IFN-α *in vitro* produced significantly more IL-1ra, compared to non-stimulated cells (Fig. 6A). Next, we asked whether deficiency of Type I IFNs results in differential production of inflammatory cytokines by LMNCs. *In vivo* priming of mice with TLR9+TLR2 ligands sensitized LMNCs to *ex vivo* stimulation with LPS and resulted in significantly greater induction of inflammatory cytokines TNF-α (Fig. 6B) and IL-1β (Fig. 6C), compared with LPS-stimulated LMNCs of non-sensitized mice. In contrast to WT mice, LMNCs isolated from IRF7- and IFNAR-deficient mice showed significantly increased

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induction of TNF-α upon *ex vivo* stimulation with LPS (Fig. 6B); induction of IL-1β did not significantly differ between genotypes (Fig. 6C).

Consequently, we asked whether IL-1ra could play a protective role in hepatocyte death induced by synergistic activities of TNF-α and IL-1β. Treatment of primary WT hepatocytes with TNF-α did not induce hepatocyte death (Fig. 6D), consistent with previous reports that healthy WT hepatocytes are resistant to stimulation with TNF-α alone.[27] However, pretreatment with IL-1β sensitized hepatocytes to TNF-α induced death (Fig. 6D). More important, the sensitizing effect of IL-1β was significantly reduced by IL-1ra (Fig. 6D). Stimulation with IL-1β + TNF-α lead to a significantly greater extent of hepatocyte death in IRF7- and IFNAR1-deficient hepatocytes, compared to WT; significant protection from hepatocyte death was observed after co-treatment with IL-1ra in all genotypes (Fig. 6E).

Taken together, these data suggested that Type I IFNs induce IL-1ra production both in hepatocytes and LMNCs, and that IL-1ra protects hepatocytes from IL-1β-dependent sensitization to TNF-α-induced cell death.

Type I interferon induces endogenous IL-1ra in vivo and ameliorates TLR9-associated liver injury

To evaluate the protective role of Type I IFN in TLR9-associated liver injury, we first administered pegylated IFNα2a (pegIFNα2) to WT mice and observed a significant induction of serum IL-1ra (Suppl. Fig. 2). Next, we showed that pegIFNα2 administered to TLR9+TLR2-primed mice two hours prior to LPS significantly prolonged survival (Fig. 7A) and ameliorated

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TLR9-associated liver injury (Fig. 7B), compared to control mice. Furthermore, pegIFNα2 prevented aggravation of LPS-induced liver injury after TLR9+TLR2-priming (Fig. 7B). Importantly, serum IL-1ra positively correlated with the length of survival (Suppl. Fig. 3A), and negatively correlated with the extent of liver injury (Suppl. Fig. 3B). Accordingly, histopathology analysis revealed reduction in liver inflammatory infiltrate and the extent of necrosis in mice pretreated with pegIFNα2 compared to controls (Fig. 7C). Taken together, these data suggest that type I IFNs induce IL-1ra which has hepatoprotective and anti-inflammatory effects in TLR9-induced liver injury.

IL-1ra ameliorates TLR9-associated liver injury

To demonstrate the direct protective effect of IL-1ra, we initiated treatment with recombinant IL-1ra in WT mice 24 hours prior to administration of TLR9+TLR2 ligands and continued with IL-1ra throughout the experiment. Mice pretreated with IL-1ra showed significantly prolonged survival after injection of LPS (Fig. 7D), which was associated with lower ALT levels (Fig. 7E) and reduction in liver inflammatory infiltrate and extent of necrosis compared to controls (Fig. 7F). Collectively, these data suggested that Type I IFNs are needed to elicit a strong anti-inflammatory response by induction of IL-1ra, and that Type I IFN-regulated IL-1β/IL-1ra system is key to the protective role of Type I IFNs in liver injury.

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Discussion

TLR9-dependent liver damage is involved in the pathogenesis of several liver diseases, such as alcoholic liver disease,[5] primary biliary cirrhosis,[6] primary sclerosing cholangitis[7] and acetaminophen-induced liver injury,[8] as well as in pathological processes such as liver fibrosis,[10] liver cirrhosis,[11] ischemia-reperfusion injury,[12] and liver graft rejection.[13] Thus, TLR9 holds the common link between different processes which lead to liver diseases; therefore, unraveling the pathogenesis of TLR9-induced liver injury may aid in identification of novel, efficient pathogenesis-based management or cure. Here we report the novel finding that Type I IFNs have an important regulatory role in TLR9-induced liver injury by limiting inflammation and liver injury. Further, we demonstrate that Type I IFN-induced IL-1ra could serve as a potential therapeutic target in liver injury.

In the current liver injury model, three TLR agonists are being used. Our recent and previous [15, 16] data show that liver inflammation and injury induced in this model are primarily dependent on TLR9; liver damage elicited by CpG is aggravated by co-stimulation with TLR2 and a secondary stimulation with TLR4. Of these TLRs, TLR2, 4 and 9 activate the MyD88-dependent pathway, while IRF3 activation is exclusive to TLR4. Type I IFN and IRF7 induction can occur via both TLR9 and TLR4. [18]

We first demonstrated that Type I IFNs, IFNα and IFNß, were induced at the mRNA level in the TLR9-associated liver injury. This Type I IFN was biologically active as suggested by the increased expression of the IFN-inducible genes, ISG15 and IP-10 in the liver. Our novel observation of the increased liver inflammatory infiltrate and exacerbated liver injury in IRF7 and IFNAR1-deficient mice suggested a protective role for the Type I IFN induction in the liver.

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Indeed, previous studies suggested that Type-I IFNs can mediate anti-inflammatory effects (summarized in [28]).

Compared to IFNAR1-deficient mice, where the protective Type I IFN signaling was fully inhibited, IRF7-deficient mice could still produce TLR4-induced Type I IFNs (preferentially IFNβ) via the TLR4-IRF3 pathway. In our study, TLR9-associated liver injury was associated with induction of IFNα, IFNβ and interferon-stimulated genes in wild-type mice. Notably, IFNAR1-deficiency abrogated the induction of Type I IFN response to much greater extent than IRF7 deficiency. Also, IFNAR1-deficient mice showed the most severe TLR9-associated histological liver damage, which exceeded the extent present in IRF7-deficient mice. The different observation between IRF7- and IFNAR1-deficient mice could be explained by differential composition of inflammatory liver infiltrate, or by the fact that Type I IFNs are potentially inducible by multiple pathways, including IRF1, IRF3 and IRF7.[23, 29] Therefore, protective Type I IFNs could still be induced in IRF7-deficient mice by TLR9 in an IRF7-independent, IRF3-dependent manner, while the downstream protective effect of Type I IFNs is completely abolished in IFNAR1-deficient mice which cannot respond to type I IFNs.

We found that deficient Type I IFN signaling was associated with decreased liver recruitment of dendritic cells and with decreased expression of CCL-21and CCR7, a key chemokine-receptor pair involved in dendritic cell trafficking.[30] Our novel findings indicating a role of Type I IFNs in recruitment of dendritic cells and in expression of CCL-21, together with data showing that TLR9-activated dendritic cells are key producers of Type I IFNs,[31] suggest a novel self-sustaining mechanism for dendritic cell recruitment into the liver. It has been reported that dendritic cells are essential for Th1 response in the liver induced by TLR9 + TLR2 ligands [32] and

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contribute to liver fibrosis,[9] Consistent with earlier observations[15] TLR9-induced inflammatory infiltrates, that require dendritic cell recruitment to the liver,[32] amplified pro-inflammatory cytokine induction by LPS. Our novel data suggest a dual role for dendritic cells in the liver. First, dendritic cells are necessary for TLR9-induced inflammatory cell infiltrates and sensitization to TLR4 ligands. Second, dendritic cells provide anti-inflammatory signals mediated by Type I IFNs.

We observed a synergistic effect of TLR9 + TLR2 and TLR4 ligands in upregulation of IL-1ra in wild-type animals; both IRF7-deficient and IFNAR1-deficient mice showed decreased induction of IL-1ra, compared to WT. One can speculate that this synergistic effect of TLR ligands on IL-1ra induction is mediated by Type I IFNs and IRF7. This notion is consistent with our data showing a synergistic effect of TLR9+2 and TLR4 ligands on Type I IFN expression in wild-type mice. We observed that Type I IFN induces IL-1ra in hepatocytes and liver mononuclear cells, and that IL-1ra protects hepatocytes against the sensitizing effect of IL-1β towards cytotoxicity induced by TNF-α. Our findings support previous reports that in order to become susceptible to the cytotoxic effect of TNF-α, hepatocytes require priming with ligands that interfere with their proliferation.[27] Indeed, IL-1β has been shown to inhibit DNA synthesis in rat hepatocytes.[33]

We further showed that administration of recombinant pegIFNα2 induced IL-1ra *in vivo*. This finding was in agreement with our *in* vitro data from hepatocytes and liver mononuclear cells, with *in vitro* data that *IL-1RA* promoter region contains Type I IFN-inducible elements,[34] and with data demonstrating increased *in vivo* production of IL-1ra upon treatment with recombinant

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IFN in humans.[35] In addition, we observed that *in vivo* administration of pegIFNα2 significantly ameliorated TLR9-associated survival, liver injury and inflammation.

Our novel data demonstrated that pretreatment with recombinant IL-1ra significantly ameliorated liver injury and inflammation in WT mice. These findings confirm the anti-inflammatory effect of IL-1ra in liver injury and are complementary to study of Iizasa et al.[36] who showed that deficiency of IL-1ra resulted in exacerbated liver injury and inflammation induced by *Propionibacterium Acnes*, which activates TLR9 and TLR2 receptors.[15, 16] Our results show for the first time that Type I IFNs potentially protect from liver injury by inducing anti-inflammatory IL-1ra.

IL-1ra acts as a natural antagonist of IL-1β,[26] and we show that in the liver IL-1ra is produced by both hepatocytes and liver mononuclear cells. Our data also demonstrates the protective effect of IL-1ra in TLR9-associated liver injury and survival. In previous studies, Imaeda et al.[8] showed that in acetaminophen hepatotoxicity, inflammatory response is triggered by apoptotic mammalian DNA that increases transcription of IL-1β, and that inflammatory response is ameliorated in mice deficient for TLR9 and in mice treated with anti-IL-1β antibody. Importantly, IL-1ra has been reported for the treatment of hepatic failure in rats using a bioartificial liver device.[37] These studies support our finding that the balance between IL-1β/IL-1ra is of crucial importance in TLR9-induced liver damage (Suppl. Fig. 4).

In conclusion, our findings suggest that the endogenous anti-inflammatory signaling induced by Type I IFNs and mediated by IL-1ra regulates the extent of TLR9-induced liver damage, and support the indispensable role of Type I interferon signaling in immune mediated liver injury. Finally, we suggest the potential role of IL-1ra in therapy of TLR9-associated liver diseases.

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Figure legends

Fig. 1. *Type I IFNs are induced in TLR9-associated liver injury*

Wild-type mice were injected i.p. with 2.5 mg/kg CpG DNA and 5 mg/kg LTA. Three days later mice were injected with saline or 0.5 mg/kg LPS i.p. and sacrificed after 2 hours. Serum ALT levels (A) were measured and messenger RNA levels of (B) liver interferon α-4 (*IFNA4*), (C) interferon β (*IFNB*) and (D) interferon-stimulated gene 15 (*ISG15*) were analyzed by real-time PCR and normalized to 18s. Values are shown as mean ± SEM fold increase over saline-primed group (3-6 mice per group). Numbers in graphs denote *p* values. *) $p < 0.05$ vs. saline-stimulated control mice; #) $p < 0.05$ vs. LPS-stimulated control mice

Fig. 2. *Deficiency of Type I IFN induction exacerbates liver injury*

B6.129F2 wild-type mice and IRF7-deficient mice were treated with CpG DNA + LTA ± LPS as in figure 1. Serum ALT levels were measured (A). Assessment of liver inflammatory infiltrate (B) was performed in histology samples stained with H&E. Arrows point at inflammatory infiltrates, magnification 200x. mRNA levels of liver (C) interferon α-4 (*IFNA4*), (D) interferon β (*IFNB*), (E) interferon-stimulated gene 15 (*ISG15*) and (F) *IP-10* were analyzed by real-time PCR. Values are shown as mean ± SEM fold increase over saline-primed control group (3-6 mice per group). *) $p < 0.05$ vs. saline-primed wild-type mice; #) $p < 0.05$ vs. saline-primed IRF7-/- mice

Fig. 3. *Deficiency of Type I IFN signaling exacerbates liver injury*

C57Bl6 wild-type mice and IFNAR1-deficient mice were treated with CpG DNA + LTA ± LPS as in figure 1. Serum ALT levels were measured (A), and H&E-stained samples assessed for

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liver inflammatory infiltrate (B) was performed in histology samples stained with H+E. Arrows point at inflammatory infiltrates, magnification 200x. mRNA levels of liver (C) interferon α-4 (*IFNA4*), (D) interferon β (*IFNB*), (E) interferon-stimulated gene 15 (*ISG15*) and (F) *IP-10* were analyzed by real-time PCR. Values are shown as mean ± SEM (3-6 mice per group). *) $p < 0.05$ vs. saline-primed wild-type mice; #) $p < 0.05$ vs. saline-primed IFNAR1-/- mice

Fig. 4. *Deficiency of Type I IFN signaling results in decreased dendritic cell recruitment to the liver in TLR9-associated liver injury*

(**A,B**) Wild-type and IFNAR1-deficient mice were injected i.p. with CpG DNA + LTA ± LPS as indicated. Liver mononuclear cells were isolated, stained with anti-CD68 (A), anti-CD11c and anti-PDCA1 (B) monoclonal antibodies and analyzed using flow cytometry (N= 5 mice per group). mRNA levels of (C) liver chemokine ligand 21 (CCL-21) and (D) chemokine receptor 7 (CCR7) were analyzed by real-time PCR. Values are shown as mean ± SEM. *) $p < 0.05$ vs. saline-primed wild-type mice; #) $p < 0.05$ vs. saline-primed IFNAR1-deficient mice

Fig. 5. *Deficient Type I interferon signaling results in an imbalance in IL-1β / IL1 receptor antagonist induction in TLR9-associated liver injury.*

Mice were treated with CpG DNA + LTA ± LPS as in figure 1. mRNA levels of liver pro-interleukin-1β (IL-1β) and interleukin 1-receptor antagonist (IL-1ra) were analyzed by real-time PCR (A,C). Serum IL-1ra levels were measured by ELISA (B,D). Values are shown as mean ± SEM fold increase over saline-primed group (3-6 mice per group). *) $p < 0.05$ vs. saline-primed wild-type B6.129F2 or C57Bl6 mice; #) $p < 0.05$ vs. saline-primed IRF7-/- or IFNAR1-/- mice

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Fig. 6. *IL-1ra protects hepatocytes from IL-1β –dependent sensitization to cell death induced by TNF-α.*

(**A**) Hepatocytes and liver mononuclear cells (LMNCs), isolated from WT mice, were stimulated with murine IFN-α2b, and IL-1ra in supernatants was measured with ELISA (N=5 mice per group). (**B,C**) WT, IRF7- and IFNAR1-deficient mice were treated with i.p. with CpG DNA + LTA. Three days later, LMNCs were isolated, *ex vivo* stimulated with LPS, and TNF-α and IL-1β in supernatant were measured after 6 hours. Representative values from total N= 4 mice per group are shown. *,#,§,†) $p < 0.05$ vs WT cells from the respective treatment group. (**D**) Primary WT hepatocytes were *ex vivo* pretreated with murine IL-1ra and IL-1β. After four hours, murine TNF-α was added. LDH release into cell culture supernatant was measured at 24 hours and normalized to total LDH. Representative values from total N= 4 mice are shown. *) $p < 0.05$ vs. cells not treated with IL-1β; #) $p < 0.05$ vs. cells not treated with IL-1ra. (**E**) Primary hepatocytes from WT, IRF7- and IFNAR1-deficient mice were isolated and treated *ex vivo* as indicated for 4 hours, followed by murine TNF-α. LDH release into cell culture supernatant was measured at 24 hours and normalized to total LDH. Representative values from total N= 4 mice per group are shown. *,#) $p < 0.05$ vs. WT hepatocytes of the respective treatment group.

Fig. 7. *Type I interferon induces endogenous IL-1ra and ameliorates TLR9-associated liver injury.*

(**A-C**) Wild-type mice were injected with CpG DNA + LTA i.p., followed 3 days later by saline or 100,000 IU pegIFNα2, and followed by LPS i.p. for the last 2 hours. Survival (A) and serum ALT were analysed at indicated time points (B). Livers were stained with H&E, magnification

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200x (C). Values are shown as mean ± SEM (11 mice per group). *) $p < 0.05$ vs. control mice, #) $p < 0.05$ vs. control mice treated with pegIFNα2.

(**D-F**) Wild-type mice were treated with saline or with recombinant IL-1ra 25 mg/kg i.p. every six hours. Twenty-four hours after IL-1ra initiation, mice were injected with CpG DNA + LTA, followed by LPS 3 days later. Survival (D) and serum ALT (E) were analysed at indicated time points. Livers were stained with H&E, magnification 200x (F). Values are shown as mean ± SEM (15 mice per group). *) $p < 0.05$ vs. control mice, #) $p < 0.05$ vs. control mice treated with IL-1ra

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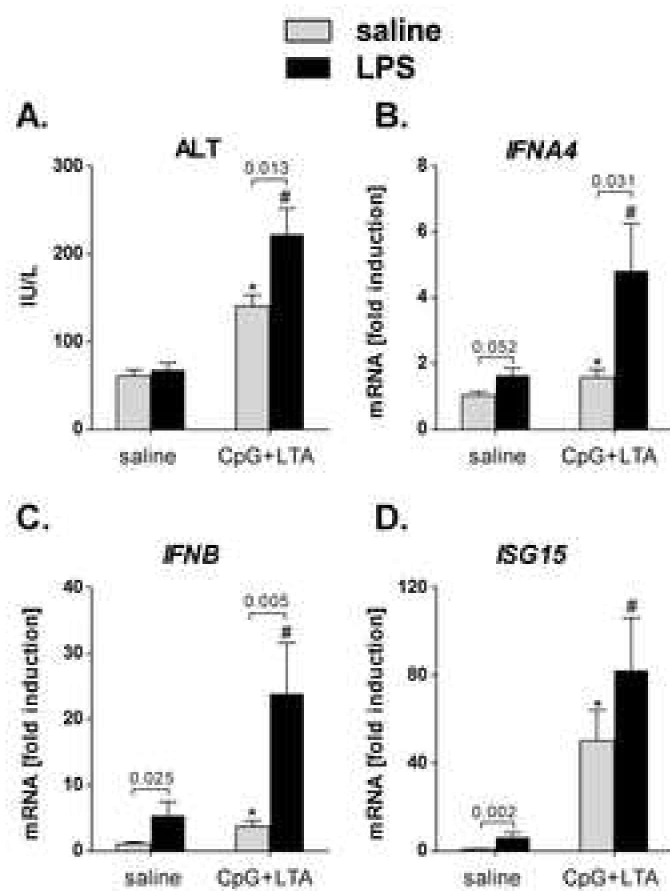

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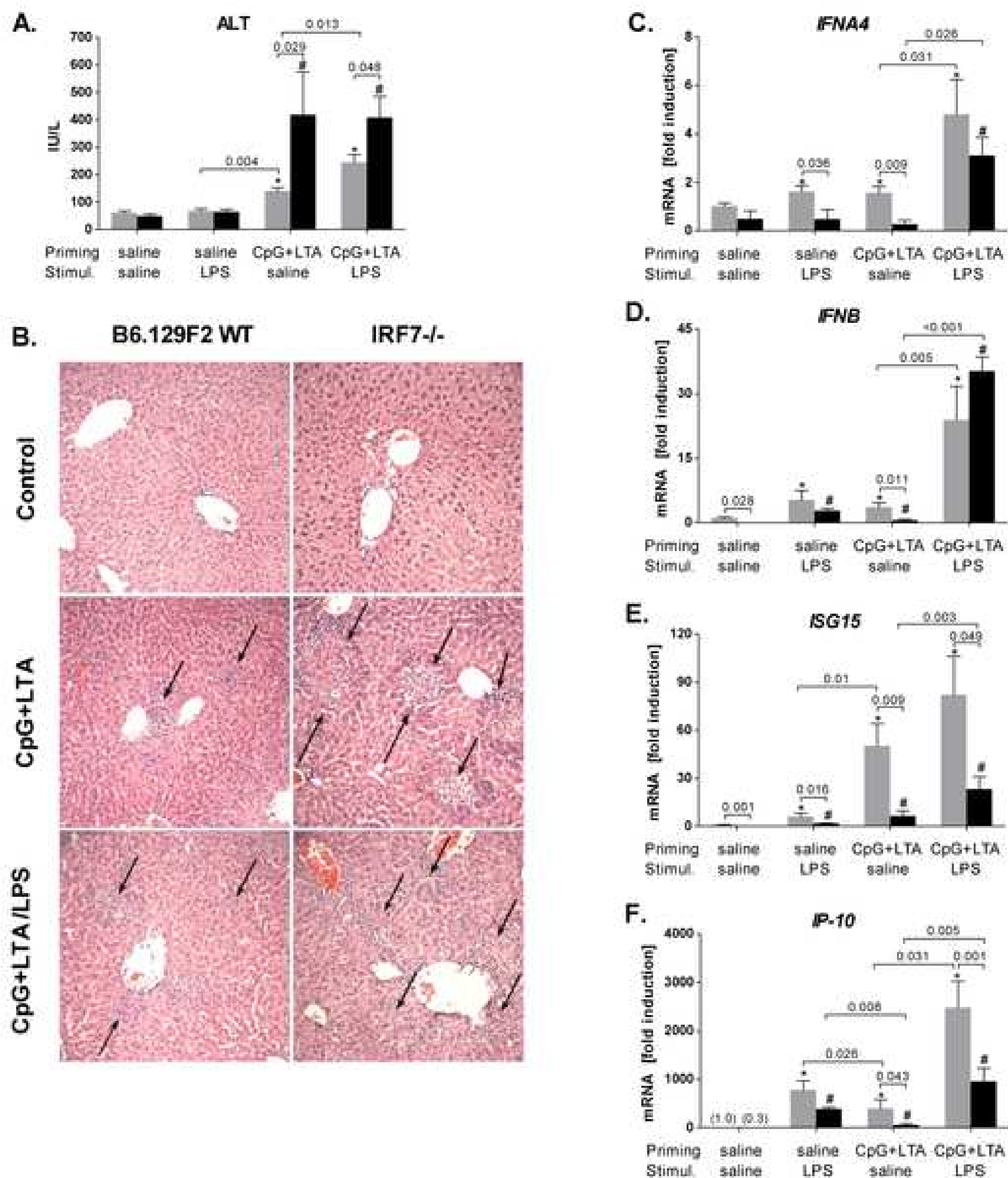

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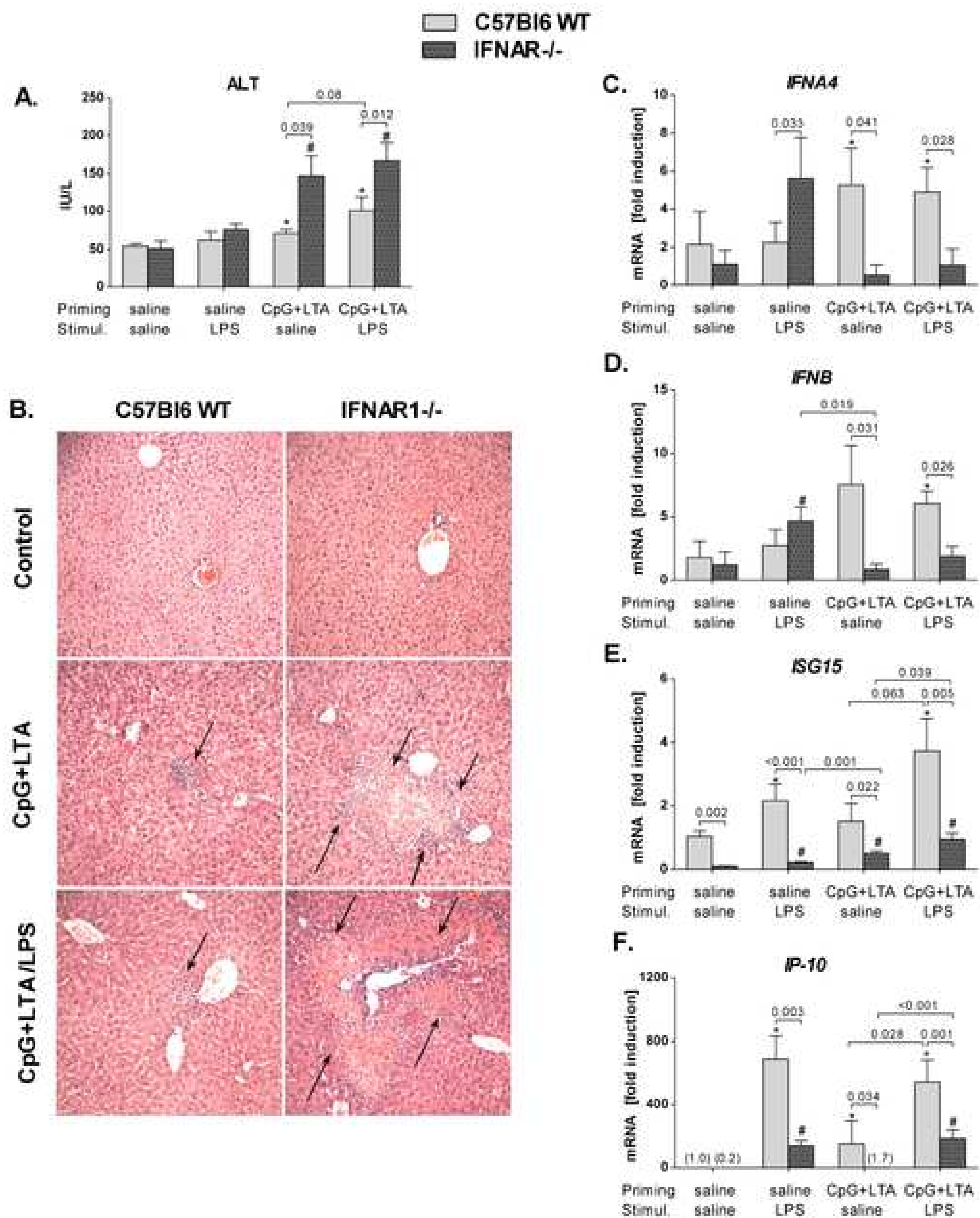

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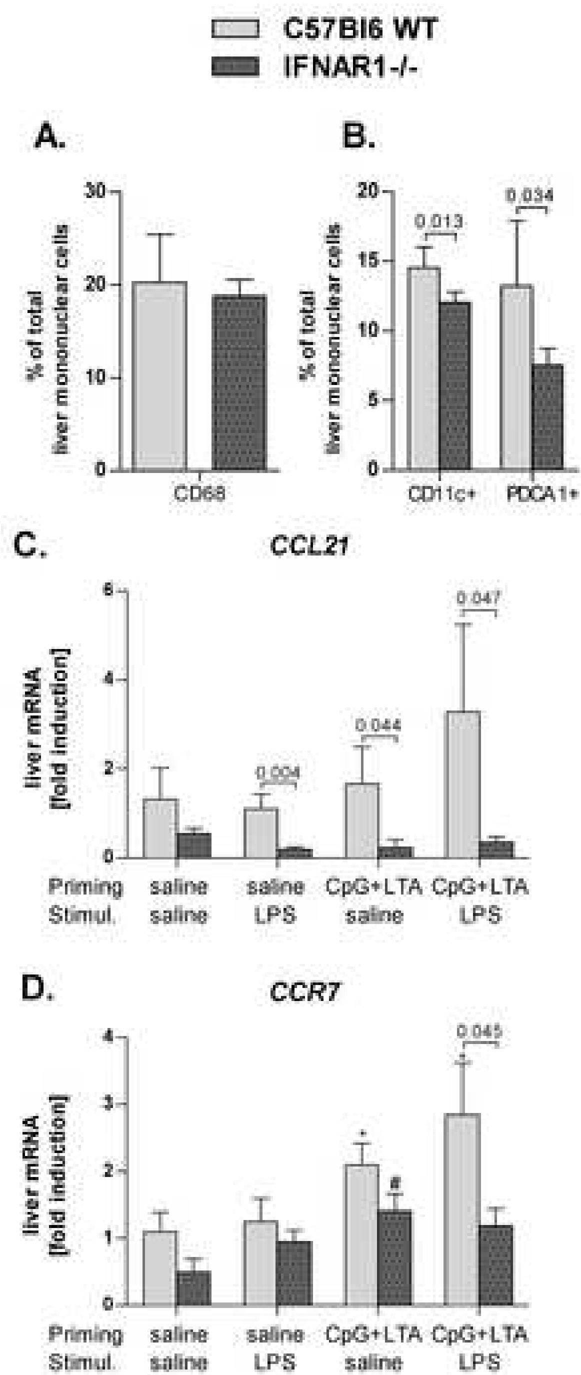

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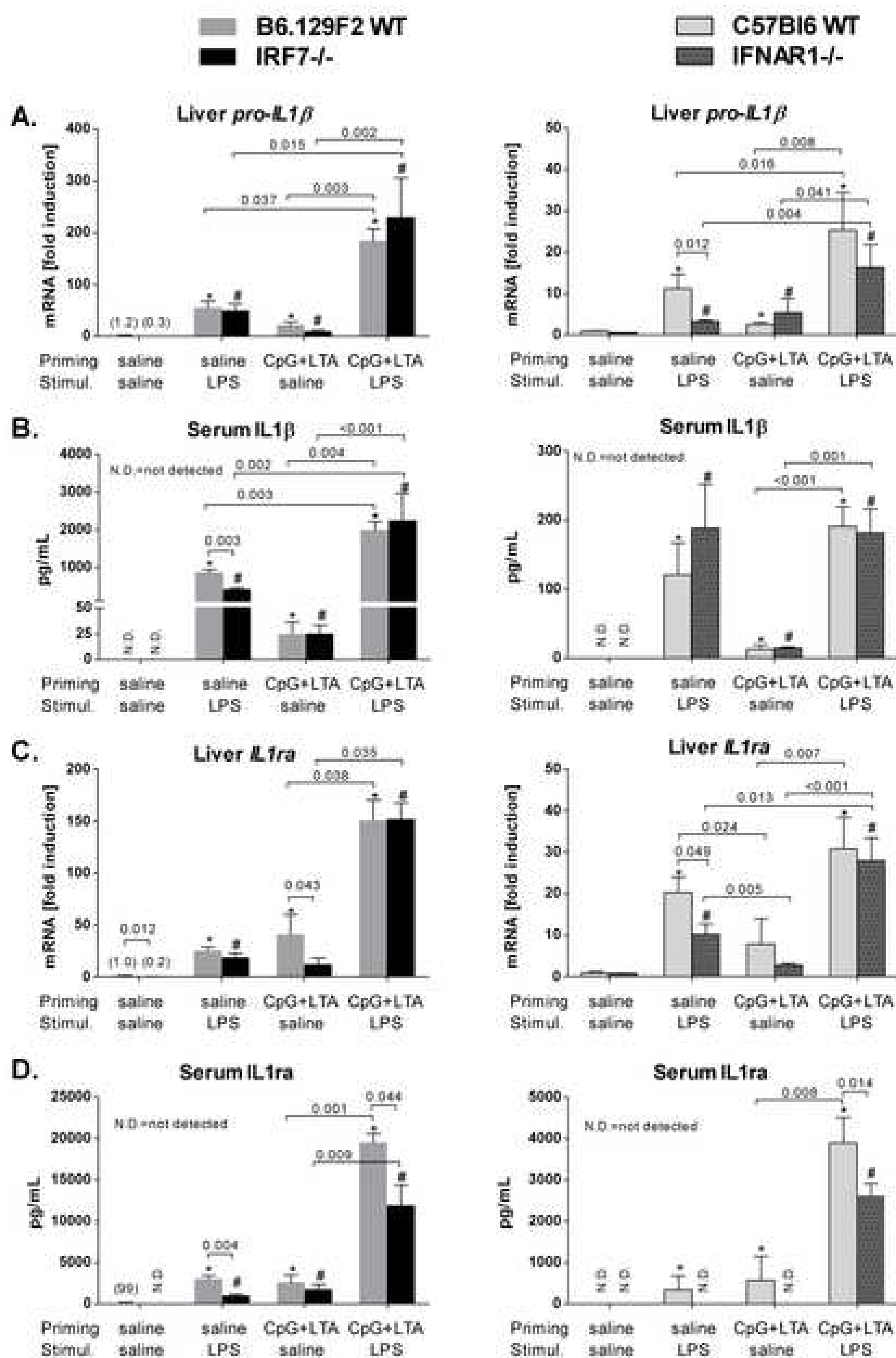

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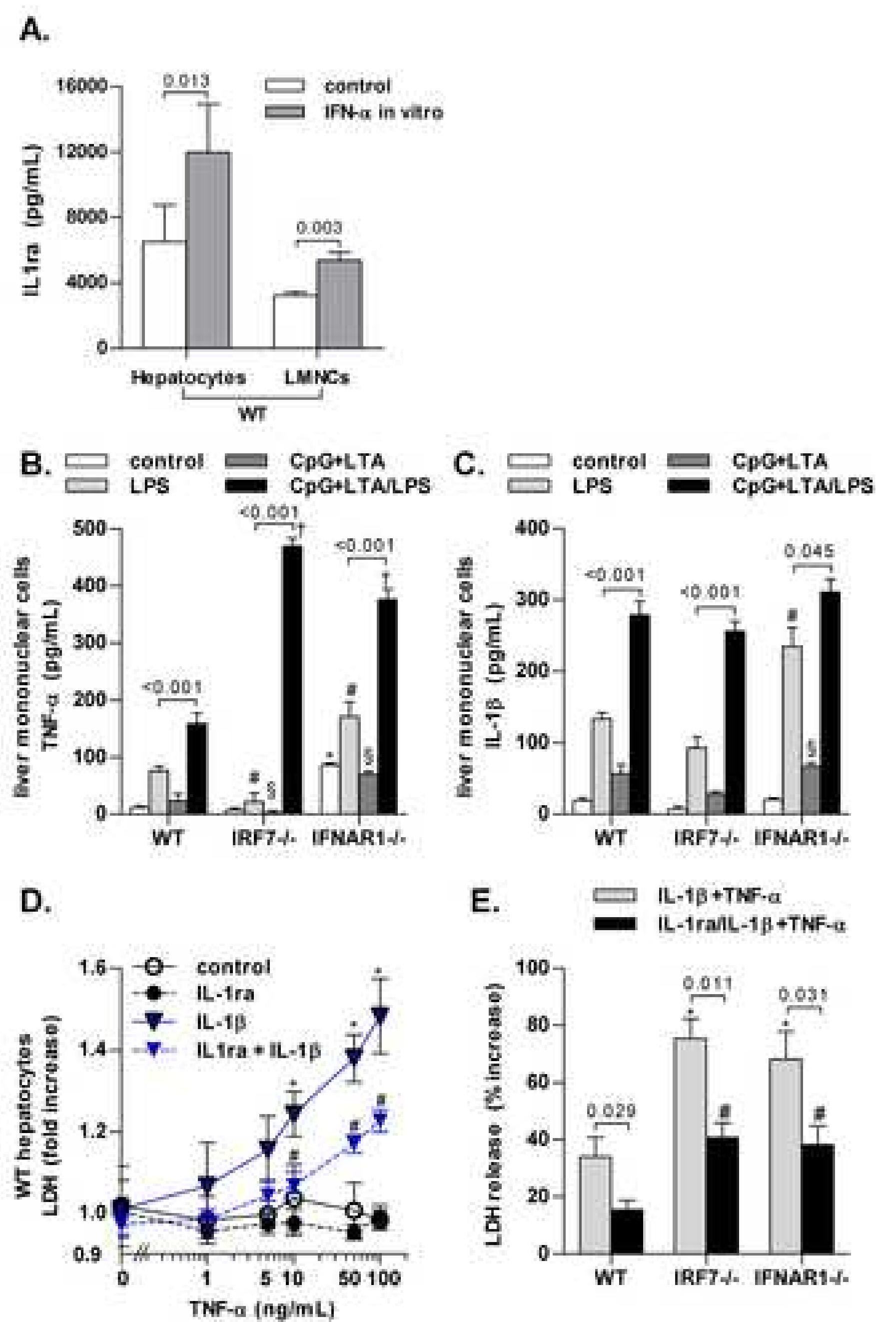

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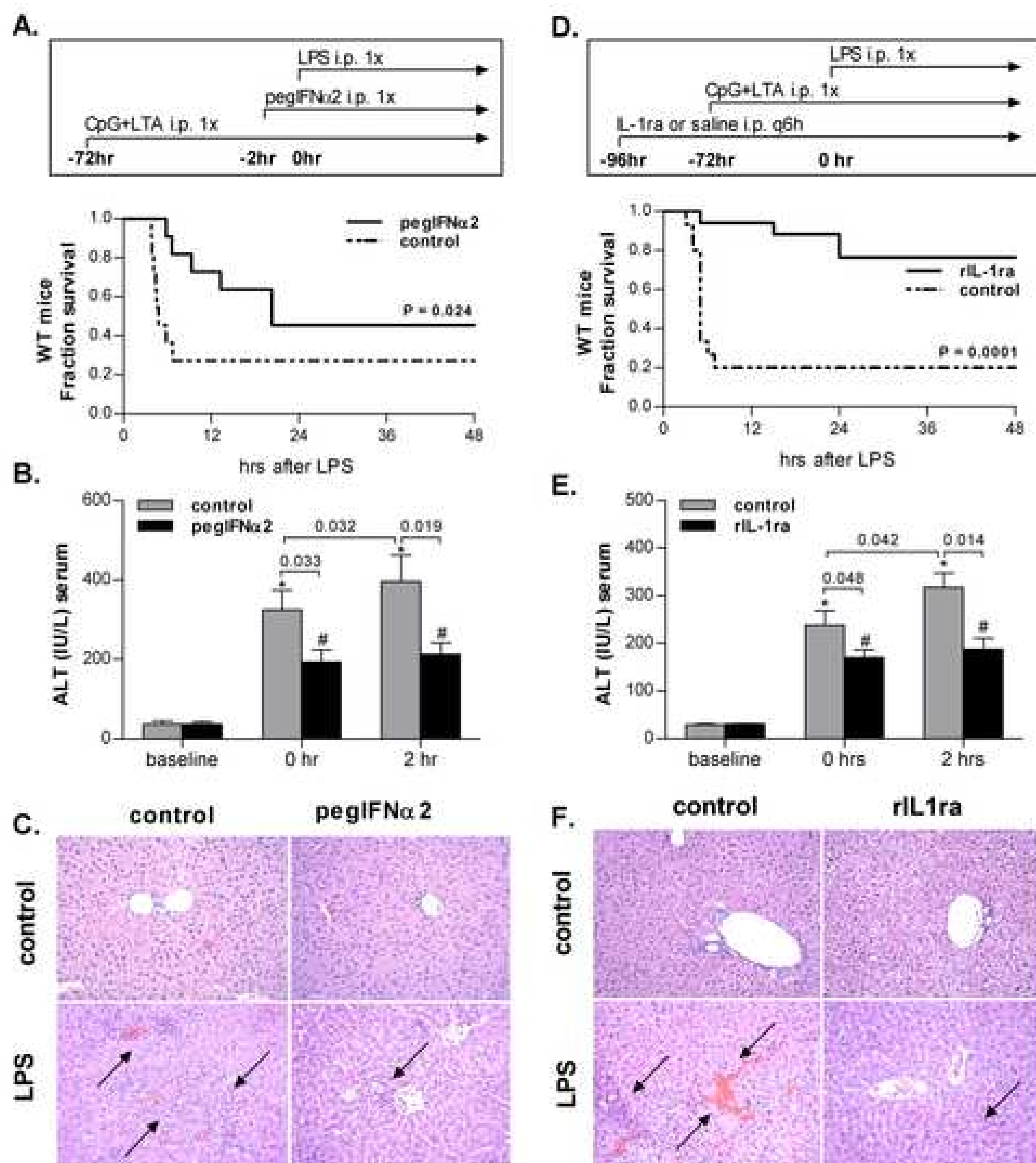

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Supplementary table 1. Real-time PCR primer sequences and reaction conditions

Primer	Sense 5'-3'	Antisense 3'-5'
18S	gta acc cgt tga acc cca tt	cca tcc aat cgg tag tag cg
pro-IL-1B	tct ttg aag ttg acg gac cc	tga gtg ata ctg cct gcc tg
IL-1RA	tca gat ctg cac tca atg cc	ctg gtg ttt gac ctg gga gt
IFNA4	agg att tgg gat tcc cct tg	tat gtc ctc aca gcc agc ag
IFNB	agc tcc aag aaa gga cga aca t	gcc ctg tag gtg agg gtt gat ct
ISG-15	cag gac ggt ctt acc ctt tcc	agg ctc gct gca gtt ctg tac
IP-10	ccc cgg tgc tgc gat gga tg	agc tga tgt gac cac ggc tgg
MCP-1	cag gtc cct gtc atg ctt ct	tct gga ccc att cct tct tg
MCP-2	cca gat aag gct cca gtc acc t	ggc act gga tat tgt tga ttc tct
MIP-1α	tct cag cgc cat atg gag ct	ttc cgg ctg tag gag aag ca
MIP-1β	ccg agc aac acc atg aag c	cca ttg gtg ct gaga acc ct
CCL-21	aaa gaa ccg gga acc tct aa	cag tcc tgc tgt ctc ctt cc
CCR1	gtt ggg acc ttg aac ctt ga	ccc aaa ggc tct tac agc ag
CCR2	agg agc ctc ttt gcc ttg tgg c	tgg cag cct cat gcc ctc ct

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CCR5	tgg ggt gga gga gca ggg ag	tag gcc aca gca tcg gcc ct
CCR7	tcc tag tgc cta tgc tgg ct	atg aag act acc acc acg gc

Real-time polymerase chain reaction (RT-PCR) was performed using the iCycler iQ Cycler (Bio-Rad Laboratories). The PCR conditions were: 95°C for 15 minutes followed by 50 cycles at 95°C for 15 seconds, 60°C for 10 seconds, and 72°C for 30 seconds. The reaction mixture for the SYBR Green assay contained 12.5 µL SYBR Green PCR Master Mix (Eurogentec, Fremont, CA), 0.5 µM of forward and reverse primer and 1 µL of complementary DNA (corresponding to 100 ng RNA) for a total volume of 25 µL. All amplifications and detections were carried out in a MicroAmp optical 96-well reaction plate with optical tape. At each cycle, accumulation of PCR products was detected by monitoring the increase in fluorescence by double-stranded DNA-binding SYBR Green. After PCR, a dissociation melting curve was constructed in the range of 55°C to 95°C. All data were analyzed using Bio-Rad iCycler software. The 18S was used for normalization of all experiments. Data was analyzed using the comparative Ct method (ΔΔCt method) using the following formula: ΔCt = Ct (target) - Ct (normalizer). The comparative ΔΔCt calculation involved finding the difference between the sample ΔCt and the baseline ΔCt. Fold increase in the expression of specific mRNA compared with 18S was calculated as $2^{-(\Delta\Delta Ct)}$.

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Supplementary figure legends

Supplementary Fig. 1. *Induction of chemokines and chemokine receptors involved in monocyte/macrophage recruitment in TLR9-associated liver injury*

C57Bl6 wild-type mice and IFNAR1-deficient mice were treated with CpG DNA+LTA ± LPS asi in figure 1. mRNA levels of liver (A) macrophage chemotactic protein 1 (*MCP-1*), (C) macrophage chemotactic protein 2 (*MCP*-2), (D) macrophage inflammatory protein 1 alpha (*MIP-1α*), (E) macrophage inflammatory protein beta (*MIP-1β*), (F) chemokine receptor 1 (*CCR1*), (G) chemokine receptor 2 (*CCR2*) and (H) chemokine receptor 5 (*CCR5*) were analyzed by real-time PCR. Liver MCP-1 protein (B) was measured using ELISA. Significantly decreased liver expression of MCP1, MCP2, MIP-1α and MIP-1β was observed in IFNAR1-mice stimulated with LPS, compared to WT mice. Values are shown as mean ± SEM (3-6 mice per group). *) $p < 0.05$ vs. saline-primed wild-type mice; #) $p < 0.05$ vs. saline-primed IFNAR1-deficient mice

Supplementary Fig. 2. *Induction of endogenous IL-1ra by Type I IFNs in vivo*

Wild-type mice were injected with 100,000 IU pegIFNα2 i.p. and IL-1ra in serum was measured at indicated timepoints (N = 4 mice). Numbers denote *p* values compared to baseline.

Supplementary Fig. 3. *Serum IL-1ra positively correlates with survival and negatively correlates with liver damage in TLR9-associated liver injury.*

Wild-type mice were injected with CpG DNA + LTA i.p. Three days later mice received saline or pegIFNα2, followed by LPS i.p. for the last 2 hours. Serum IL-1ra at was correlated with the length of survival (A) and serum ALT (B). N = 6-7 mice per group.

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Supplementary Fig. 4. *Proposed mechanism of the protective role of Type I IFNs in TLR9-associated liver injury.*

TLR9, TLR2 and TLR4 ligands stimulate liver macrophages to produce inflammatory cytokines. TLR9 ligands stimulate dendritic cells to produce Type I IFNs in IRF7-dependent manner. Type I IFNs bind to Type I IFN receptors (IFNAR1) and induce IL-1ra in hepatocytes and in liver mononuclear cells. IL-1ra binds to IL-1 receptor (IL-1R) and inhibits IL-1β –dependent sensitization to TNF-α –induced hepatocyte death. Blue and black arrows depict interaction pathways; red arrows depict pathways of potential therapeutic targets

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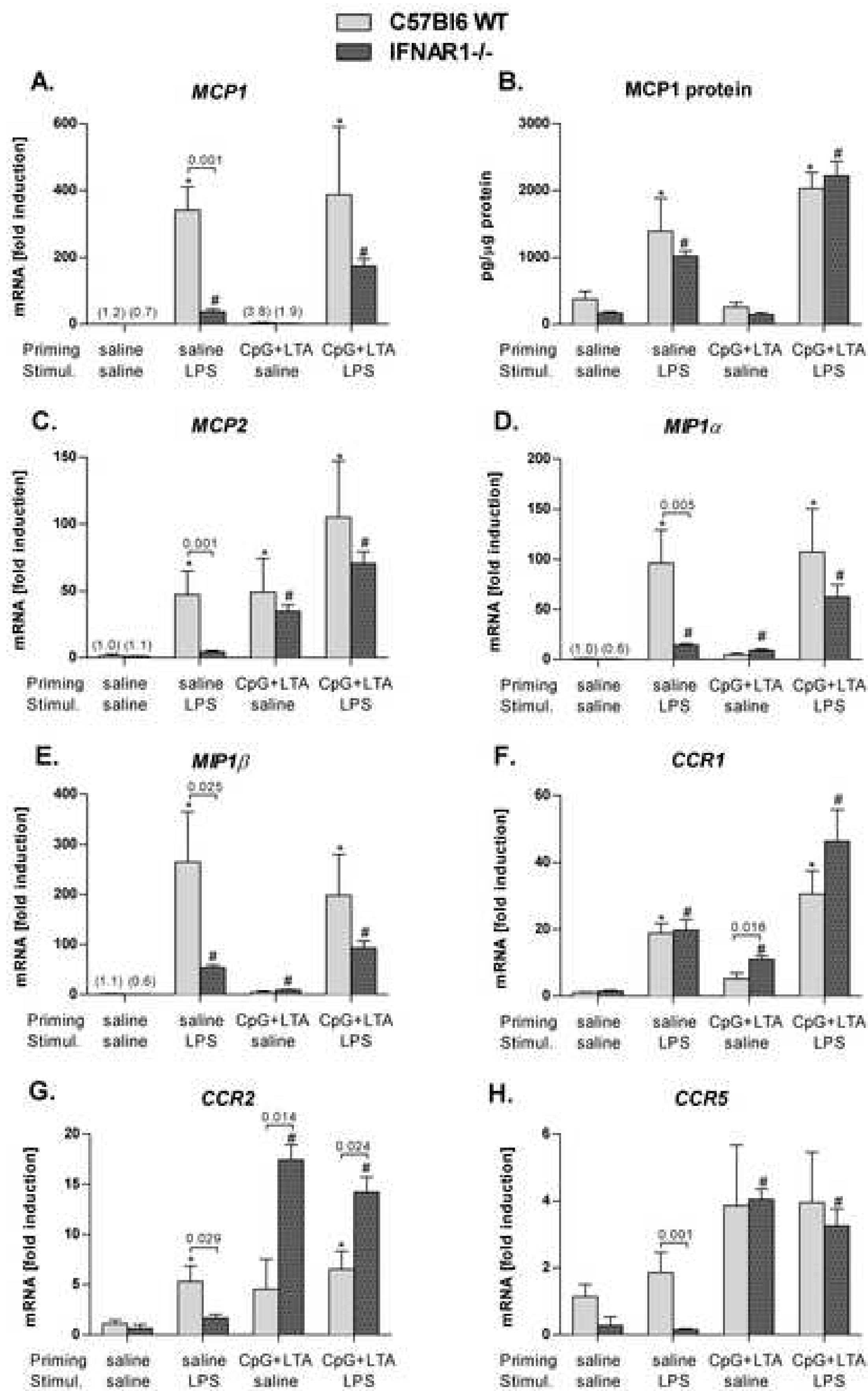

Supplementary Figure 1.

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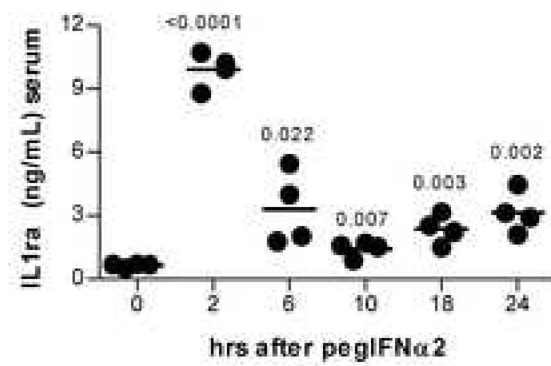

Supplementary Figure 2.

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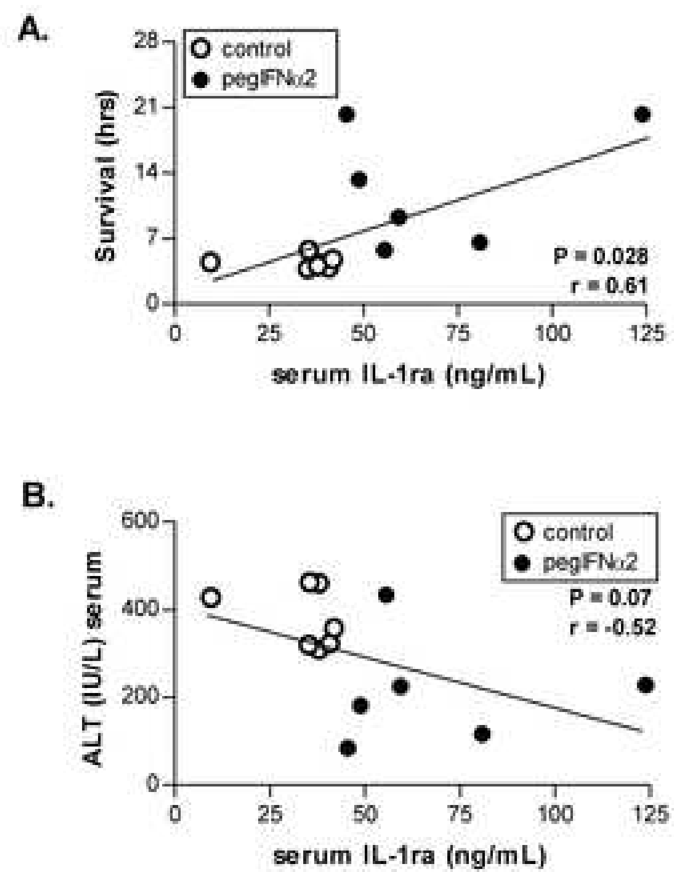

Supplementary Figure 3.

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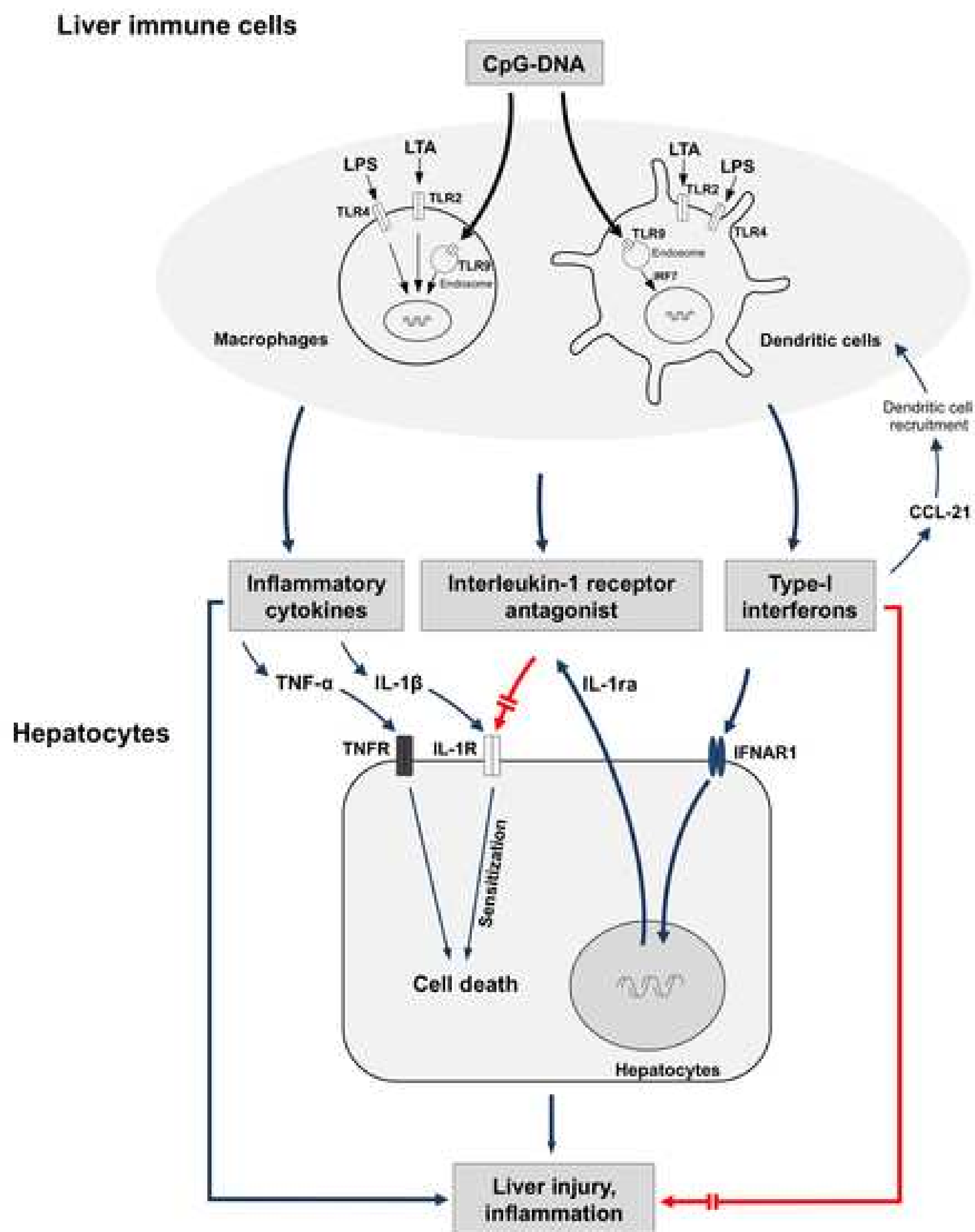

Supplementary Figure 4.