The role of liver p38α in acute and chronic inflammatory reactions

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität zu Köln

vorgelegt von

Jan Heinrichsdorff

Köln 2010

Berichterstatter: Prof. Manolis Pasparakis

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Tag der mündlichen Prüfung: 25.10.2010

Part of this work was published in the following journal:

Heinrichsdorff, J., T. Luedde, et al. (2008). "p38 alpha MAPK inhibits JNK activation and collaborates with IkappaB kinase 2 to prevent endotoxin-induced liver failure." EMBO Rep **9**(10): 1048-54.

Acknowledgments

During my four years as a member of the Pasparakis Lab, I have acquired a vast range of experiences and knowledge, not only in a scientific manner, but also personally. These experiences have allowed me to grow and become more mature in many aspects of my life. For this reason, I would like to thank all the people from inside and outside the Pasparakis lab that contributed in making these four years a life changing experience.

Most of all, I would like to thank Manolis, for giving me the opportunity to perform my PhD work in his laboratory. Manolis is a great supervisor that on the one hand demands a high degree of commitment, but is also rewarding and permitting the acquisition of a vast range of knowledge and skills through the many resources available in his lab. I strongly believe that during my time in this laboratory I have learned what it really means to be a scientist performing basic research.

Furthermore, I would like to thank Dr. Tom Lüdde and Dr. Hanno Ehlken who, through excellent supervision at the beginning but also throughout the duration of my PhD, guided me in the right direction concerning my research projects. In addition, I would like to thank all the members of the laboratory, for valuable discussions and technical help.

I would also like to thank my parents for giving me great support throughout the whole time of my PhD work.

Die Rolle der MAP Kinase p38α in akuten und chronischen Entzündungsreaktionen

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Die Leber spielt eine wichtige Rolle für das Gleichgewicht im Körper da sie wichtige Stoffwechselfunktionen ausübt. Neben synthetischen und exkretorischen Funktionen hat die Leber auch wichtige immunologische Aufgaben. Diese Funktionen werden vor allem von Zytokinen gesteuert. Während der nuclear factor-kB (NF-κB) Signalweg vor allem anti apoptotische Funktionen hat, und Zellen vor TNF induziertem Tod schützt, hat der c-Jun amino-terminal kinase (JNK) Signalweg proapoptotische Funktionen.

Die Kinase p38α ist ein Mitglied Familie der MAP Kinasen und wird durch diverse entzündliche Reaktionen aktiviert. Darüber hinaus wurde gezeigt, dass p38α eine Funktion in der Zellproliferation, in der Differenzierung von Zellen und beim programmierten Zelltod hat. Ziel dieser Promotionsarbeit war es, die Rolle von p38α in akuten und chronisch entzündlichen Reaktionen in der Leber zu untersuchen. Hierzu wurden leberspezifische p38α knockout Mäuse generiert und in einem Model LPS/TNF induzierter akuter Leberentzündung sowie einem Model von Diät induzierter Fettleibigkeit untersucht.

Nach LPS Injektion führt die leberspezifische Deletion von p38α zu einer verstärkten Aktivierung der MAP Kinase JNK. Die verstärkte Aktivierung von JNK führt nicht zu einer erhöhten Sensitivität für LPS/TNF induzierte Leberschädigung. Im Gegensatz dazu, führt eine LPS Injektion in Mäusen mit einer leberspezifischen Deletion für p38α und die IκB kinase 2 (IKK2) zu akuten Leberversagen. Folglich führt eine partielle Inhibierung von NF-κB in Kombination mit p38α Defizienz zu akuter Leberschädigung. Die Ergebnisse zeigen eine neue Rolle von p38α das zusammen mit IKK2 LPS/TNF induzierte Leberschädigungen verhindert.

Darüber hinaus führt die Deletion von p38α in der Leber zu erhöhter Insulinresistenz in einem Model von Diät induzierter Fettleibigkeit und Insulinresistenz. p38α defiziente Lebern und primäre Hepatozyten zeigen eine Einschränkung von Insulin vermittelten Signalen. Dagegen hat der p38α Knockout keinen Einfluss auf Quantität und Qualität von Leberlipiden. Auch eine erhöhte Expression von entzündlichen Zytokinen in der Leber wurde nicht detektiert. Eine erhöhte Aktivität von JNK und seinen Aktivatoren MKK4, TAK1 und ASK1 scheint für die eingeschränkte Ansprechbarkeit von p38α defizienten Zellen durch Insulin verantwortlich zu sein.
Folglich scheint die Kinase p38α in der Leber eine wichtige Funktion bei der Regulation der Glukosehomeostase zu haben, da sie eine Reihe von Kinasen reguliert die negativen Einfluss auf Insulin vermittelte Signale nehmen können.

The role of liver p38α MAP kinase during acute and chronic inflammatory states

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The liver plays an important role in body homeostasis due to its inflammatory, metabolic and synthetic functions. Cytokines control these functions by activating various intracellular signaling pathways. Activation of the nuclear factor-kB (NF-κB) pathway protects cells from TNF-induced apoptosis by inducing the expression of anti-apoptotic proteins, while sustained c-Jun amino-terminal kinase (JNK) is important for TNF-induced killing of NF-κB-deficient cells.

The p38 mitogen-activated protein kinase (p38) is a member of the MAP kinase family and is activated by various inflammatory stimuli. p38 was shown to regulate cellular responses to stress and is implicated in cell proliferation, differentiation and apoptosis. To investigate the function of p38α in the liver during acute and chronic inflammatory conditions, mice with a liver parenchymal cell specific ablation of p38α were generated. Two models of inflammation, a model of acute LPS/TNF induced liver toxicity and a model of diet induced obesity were applied.

Upon challenge with bacterial lipopolysaccharide (LPS), liver parenchymal cell specific ablation of p38α results in excessive activation of JNK. Despite increased JNK activity, p38α deficient hepatocytes are not sensitized to LPS/TNF induced apoptosis, suggesting that JNK activation is not sufficient to mediate TNF-induced liver damage. By contrast, LPS injection caused liver failure in mice lacking both p38α and IκB kinase 2 (IKK2) in liver parenchymal cells through decreased level of the anti apoptotic molecule c-FLIP (L). Thus, hyperactivation of JNK combined with partial inhibition of NF- κB sensitizes the liver to cytokine-induced damage, revealing a new function of p38α in collaborating with IKK2 to protect the liver from LPS/TNF-induced failure.

Furthermore, deletion of the p38α MAPK in liver parenchymal cells results in increased insulin resistance in obese mice. p38α deficient livers and isolated primary hepatocytes show increased insulin resistance while liver lipids remain unaffected ruling out a possible accumulation of lipid intermediates that could negatively regulate insulin signaling. Moreover, an elevated expression of inflammatory cytokines was not detected. Increased activation of kinases including JNK, MKK4, TAK1 and ASK1 is likely to be responsible for the increased insulin resistance in p38α deficient hepatocytes. Thus, in obese mice, liver p38α might have an important function regulating glucose homeostasis by controlling the activity of kinases that might be responsible for the negative regulation of insulin signaling.

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Abbreviations

Acyl-CoA	Acyl-Coenzym A
AKT	Proteinkinase B
ALFP	alpha fetoprotein
AP-1	activator protein 1
ASK	Apoptosis Signal Regulating Kinase
Atf2	activating transcription factor
Atf2	Activating transcription factor 2
ATP	Adenosine Triphosphate
BAFF	cell activating factor
BHA	butylated hydroxyanisole
BSA	Bovine Serum Albumin
CaCl2	Calcium Chloride
cDNA	Complementary DNA
c-Flip	cellular FLICE-inhibitory protein
Cre	Cyclization Recombination
CREB	response element-binding protein
DAG	Diacylglyceride
DANN	Deoxyribonucleic Acid
DAPI	4'-6-Diamidino-2-phenylindole
DD	Death Domain
DEN	diethylnitrosamine
DLK	leucine zipper-bearing kinase
DMEM	Dulbecco’s Modified Eagle Medium
DTT	Dithiothreitol
dUTP	Deoxyuridine Triphosphate
EDTA	Ethylene Diamine Tetraacetate
eIF-4E	eukaryotic initiation factor-4e
ELISA	Enzyme-linked Immunosorbent Assay
EMSA	Electromobility shift assay
ER	endoplasmatic reticulum
ERK	extracellular signal related kinases
FADD	Fas Associated Death Domain protein
FCS	Fetal Calf Serum
FRT	FLP recombinase target
GalN	D-Galactosamine

GSK3	Glykogen Synthase Kinase 3
GTT	Glucose tolerance test
H&E	Hematoxylin & Eosin
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic Acid
HFD	high fat diet
HRP	Horseradish Peroxidase
HSP27	shock protein 27
IKK1	IκB kinase 1
IKK2	IκB kinase 2
IL-1	interleukin-1
IL-6	interleukin 6
IR	Insulin receptor
IRS	insulin receptor substrate
ITT	Insulin tolerance test
IVC	individual ventilated cages
IκB	inhibitor of κB protein
JIP	JNK Interacting Proteins
JNK	c-Jun N-terminal kinase
KCL	Potassium Chloride
kDa	Kilo Dalton
KO	Knockout
LoxP	locus of X-over P1
LPA	Lysophosphatidic acid
LPC-KO	liver parchenchymal cell speciffic knockout
LPS	lipopolysaccharide
MAP3K	MAP Kinase Kinase Kinase
MAPK	Mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MK2	kinase- activated protein kinase 2
MKP	MAP kinase phospahatase
MLK	Mixed Lineage kinases
mRNA	Messenger RNA
mTOR	mammalian Target of Rapamycin
NaCL	Natrium Chloride
NaCL	Sodium Chloride
NaF	Sodium Fluoride
natural killer	NK

NCD	normal chow diet
NEMO	nuclear factor-kappa B essential modulator
NF-kB	nuclear factor-kappaB
NMR	Nuclear magnetic resonance
P	Phospho
PA	Palmatic acic
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
RIP1	Receptor Interacting Protein 1
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
Rpm	Rounds per Minute
RT	Reverse transcriptase
RT	Room Temperature
Sap1	SRF accessory protein
TAK1	TGF-b-activated kinase 1
TAO	Thousand and one amino acid kinase
TBS	Tris buffered saline
TCF	Ternary Complex Factor
TNFR II	TNF-receptor II
TNFR-I	TNF-receptor I
Tpl-2	Tumor progression locus-2
TRADD	TNFR I Associated Death Domain protein
TRAF2	TNF Receptor Associated Factor 2
TUNEL	Terminal Deoxynucleotidyl Transferase Mediated dNTP Nick End Labelling
TNF	tumor-necrosis factor alpha
WAT	white adipose tissue
WB	Western Blot
WT	Wild-Type

„… because the liver is a source of many diseases, and it is a noble organ that serves many other organs, almost all of them: so it suffers, it is not a small suffering, but a great manifold one“

Paracelsus (1494-1541)

1. Introduction

1.1 The liver

The liver is the largest solid organ present in vertebrates. It has a wide range of functions keeping the body in homeostasis. The liver plays a major role in glucose and glycogen metabolism, lipid and lipoprotein synthesis, hormone production, decomposition of red blood cells and detoxification. It produces bile, an alkaline compound which aids in digestion and detoxification, via the emulsification of lipids. The liver also performs and regulates a wide variety of high-volume biochemical reactions requiring highly specialized tissues, including the synthesis and breakdown of small and complex molecules, many of which are necessary for normal vital functions. It is absolutely essential for survival; there is currently no way to compensate for the absence of liver function as opposed to - for example – blood dialysis in kidney dysfunction.

With respect to its anatomical location the liver has a unique position in the body. Situated below the diaphragm in the thoracic region of the abdomen it allows continuous blood flow from the gastrointestinal tract through the sinusoids. A human liver consists of 2 lobes, comprising 1/50 of the body weight of an adult. In contrast, a mouse liver consists of 4 lobes (Martins, Theruvath et al. 2008).

In terms of its cellular composition, the liver is a heterogeneous organ. Circulating blood cells, from the innate or adaptive immune system and a variety of intrahepatic cell populations such as parenchymal liver cells (hepatocytes 60-65% and biliary epithelial cells 3,5% off all liver cells), the sinusoidal cell compartment including endothelial cells (15-20%), liver-resident macrophage (Kupffer cells 8-12%), lymphocyte [mainly natural killer T (NKT) cells] populations and hepatic stellate cells (3-8%) steer the different hepatic functions (Racanelli and Rehermann 2006).

The function of the diverse cell populations in the liver is orchestered by cytokines, which are key mediators within the complex interplay of intrahepatic immune cells and hepatocytes. Cytokines can activate effector functions of immune cells, as well as intracellular signaling pathways within the hepatocytes, acting as key players controlling cellular homeostasis. Serving as autocrine, paracrine or endocrine messengers, cytokines are essential mediators that influence various liver functions including inflammatory and metabolic responses (Luedde, Liedtke et al. 2002).

In the liver, Kupffer cells and liver-infiltrating monocyte-derived macrophages are primary sources of cytokines, such as tumor-necrosis factor alpha (TNF) and Interleukin-6 (IL-6). Especially TNF seems to play an outstanding role controlling liver homeostasis and is therefore involved in important functions in liver physiology and pathology. TNF activates specific intracellular pathways in hepatocytes that influence cell fate in different manners, through the activation of proapoptotic signals via caspase or Mitogen-activated protein kinase (MAPK) cascades, but also survival pathways, like the nuclear factor-kappaB (NF-κB) pathway. Recent experiments with genetically modified mice demonstrated important and partly opposing functions induced by TNF receptor (TNFR) signaling. While on the one hand TNF is able to promote cell survival and liver regeneration, on the other hand it can act as a death inducing cytokine. Therefore, the dissection of hepatocyte responses to TNF in homeostasis and injury could potentially identify novel targets for the treatment of many acute and chronic liver diseases.

1.2 Tumor Necrosis Factor

TNF was initially described 30 years ago as an endotoxin induced serum factor that induces death in BALB/c sarcomas (Carswell, Old et al. 1975). Independently, TNF was described as a factor mediating differentiation of hematopoetic cells into the monocyte/macrophage linage (Takeda, Iwamoto et al. 1986) and as a hormone produced by activated macrophages, responsible for hypertriglyceridemia and wasting of adipose tissue in cancer patients and patients suffering from infections (Beutler, Greenwald et al. 1985; Fried and Zechner 1989). It was shown to control a diverse range of physiological functions, many of which are related to the immune response and inflammation. TNF has been shown to play an important role in leukocyte migration, tissue resorption, and fever response (Beutler and Cerami 1989).

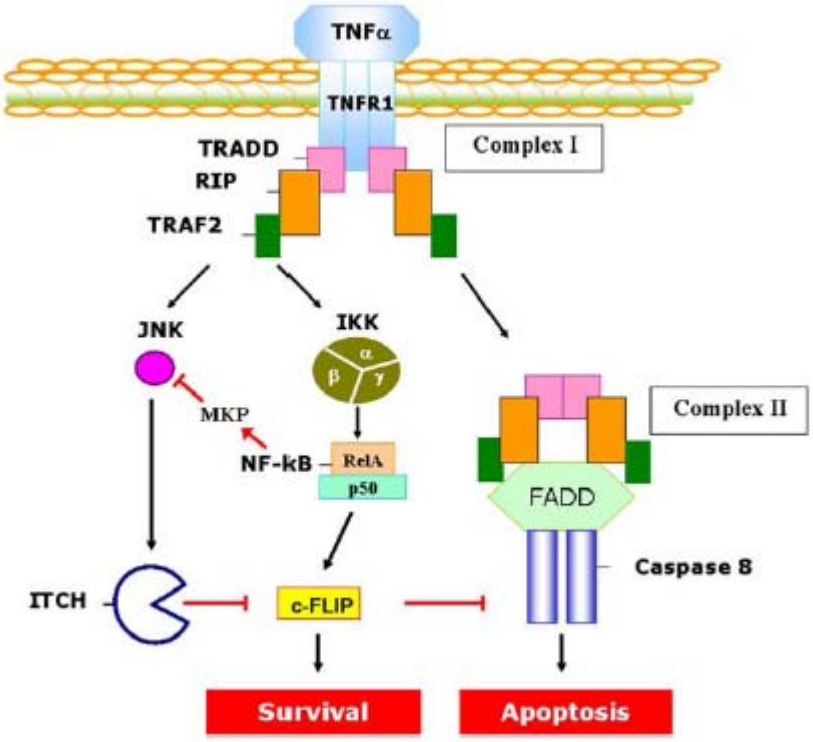
TNF binds to two plasma membrane receptors, TNF-receptor I (TNFR I) and TNF-receptor II (TNFR II) (Brockhaus, Schoenfeld et al. 1990; Dembic, Loetscher et al. 1990; Schall, Lewis et al. 1990). Whereas TNFR I is the receptor for soluble TNF, TNFR II mediates effects through the membrane bound TNF precursor and seems mostly restricted to T cells (Zheng, Fisher et al. 1995; Kim and Teh 2001; Depuydt, van Loo et al. 2005). The majority of the biological effects induced by TNF seem to be mediated by TNFR I through the activation of NF-κB and the initiation of MAPK cascades as well as cell death inducing caspase cascades. The diversity of the different pathways that are activated by TNFR I is mediated through different adaptor proteins recruited to the cytoplasmic part of the receptor. The cytoplasmic part of the TNFR I contains a Death Domain (DD) that is shared by a number of receptors promoting cell death (Tartaglia, Rothe et al. 1993). The Death Domain creates a binding

scaffold for downstream mediators of TNFR I signaling such as TNFR I Associated Death Domain protein (TRADD), Fas Associated Death Domain protein (FADD), Receptor Interacting Protein 1 (RIP1), TNF Receptor Associated Factor 2 (TRAF2) and other factors (Hsu, Xiong et al. 1995; Hsu, Huang et al. 1996; Hsu, Shu et al. 1996). According to the current model, two complexes are built upon receptor activation. Complex I leads to the activation of NF-κB, the MAP kinase pathways c-Jun N-terminal kinase (JNK) and p38. TNF-mediaded activation of NF-κB is initiated by the recruitment of the adaptor molecules TRADD, TRAF2, and Rip to the cytoplasmic part of TNFR I. Once this complex is formed, TRAF2 and RIP1 cooperate to recruit the TGFβ-activated kinase 1 (TAK1). TRAF2 contains ubiquitin ligase activity and leads to the addition of Lys-63 ubiquitin chains to RIP1. These ubiquitin chains are recognized by TAB2/3 which both mediate the recruitment of TAK1 (Chen and Goeddel 2002; Chen 2005). Activated TAK1 phosphorylates the critical residues on IKK2 and leads to the activation of NF-κB signaling (Wang, Deng et al. 2001; Sato, Sanjo et al. 2005; Shim, Xiao et al. 2005). TAK1 is a member of the MAP3K family. Therefore, recruitment of TAK1 to the TNFR I does not only cause TNF induced activation of NF-κB, it also causes activation of the MAP kinases JNK and p38. The mechanisms leading to temporal and spatial separation of MAPK and NF-κB signaling downstream of TNFR I are currently under investigation (Matsuzawa, Tseng et al. 2008). In contrast, Complex II, which forms after dissociation of the membrane proximal complex I from TNFR I, induces hepatocyte apoptosis by recruiting the adaptor molecules FADD and TRADD. FADD contains a death effector domain by which it can recruit procaspase 8. The clustering of procaspase 8 in the DISC complex leads to its autoactivation. Active caspase 8 is then able to proteolytically activate other effector caspases initiating apoptosis (Micheau and Tschopp 2003). In cells that lack substantial NF-κB activity, formation of complex II results in caspase-8 activation and apoptotic cell death (Muppidi, Tschopp et al. 2004) However, if NF-κB has been activated during complex I formation, it prevents caspase 8 activation by the DISC complex through induction of antiapoptotic genes like the cellular FLICE-inhibitory protein (c-FLIP), an adaptor protein with a pseudocaspase domain that specifically inhibits caspase-8 (Thome and Tschopp 2001). Thus, NF-κB may negatively regulate TNF induced apoptosis through regulation of c-FLIP levels and its activation serves as a checkpoint that determines whether the DISC complex will signal cell death or not (Muppidi, Tschopp et al. 2004).

1.3 TNF induced signaling in hepatocytes

In the healthy liver, cytokine production is absent or very low. During liver inflammation cells are exposed to increased levels of TNF. TNF induction is known as one of the earliest events of hepatic inflammation triggering a cascade of other cytokines that cooperate in recruiting inflammatory cells, killing of hepatocytes and initiating wound healing responses (Bradham, Plumpe et al. 1998). Depending on the cellular context, exposure of hepatocytes to TNF induces signals that mediate cell death or survival pathways, which allow hepatocytes to survive and recover from tissue damage (Akerman, Cote et al. 1992; Yamada, Kirillova et al. 1997; Yin, Wheeler et al. 1999; Rudiger and Clavien 2002). Moreover, in addition to its role in triggering life and death decisions in hepatocytes, TNF has also been shown to affect important metabolic functions in the liver. TNF expression is upregulated in the fat of obese rodents and humans and is elevated in the serum of patients with type 2 diabetes. Additionally, it has been shown to cause insulin resistance in hepatocytes *in vivo* and *in vitro* (Uysal, Wiesbrock et al. 1997; Hotamisligil 1999)

As a result of this complex interplay between different signaling cascades activated downstream of the TNFR I, there are different levels of regulation determining the biological outcome of the TNF induced signaling. Various conflicting signaling pathways lead to a wide spectrum of TNF induced responses ranging from cell survival to cell death. These signals also influence cell metabolism, gene expression, differentiation, adhesion and mortality (Shaulian and Karin 2002). Especially NF-κB and MAPK mediated responses to TNF signaling in the liver were a subject of intense investigation in the last decade.



Graph 1: TNFR I signaling TNFR1 activation leads to formation of complex I at the cytoplasmic tail of the receptor resulting in JNK1 and IKK activation. Complex I dissociates from the receptor. Complex II, which includes FADD, is formed. If c-FLIP (L) levels are high (high NF-κB, low JNK activation), caspase-8 recruitment to FADD and its subsequent activation are inhibited and the cells survive. If c-FLIP (L) levels are low (low NF-κB, high JNK), caspase-8 is recruited to FADD and activated, leading to apoptotic cell death. Image taken from Chang, L *et al.* 2006 Cell.

1.3.1 NF-κB signaling in the liver

NF-κB was identified about 20 years ago as a transcription factor that binds to the intronic enhancer of the kappa light chain gene in B cells (Sen and Baltimore 1986; Sen and Baltimore 1986). The family of NF-κB transcription factors consists of p50 and its precursor p105, p52 and its precursor p100, RelA (p65), c-Rel and RelB all of which share a Rel homology domain responsible for homo and heterodimerisation as well as for sequence specific DNA binding. RelA, c-Rel and RelB also contain a C-terminal transcription activation domain, whereas p50 and p52 do not and rely on interactions with other partners to positively regulate transcription (Hayden and Ghosh 2008). In resting cells, NF-κB transcription factors are normally kept in an inactive state in the cytoplasm through the binding to inhibitors of κB proteins IκBs (IκBα, IκBβ, IκBγ) and the precursor proteins p100 and p105.

Many different stimuli can activate NF-κB transcription factors and induce their nuclear accumulation (Ghosh and Karin 2002; Bonizzi and Karin 2004) which is controlled by the IκB kinases (IKKs).

The major and most well known pathway to activate NF-κB is the so called canonical NF-κB pathway. Activation is controlled by the IKK complex consisting of IKK1, IKK2 and the regulatory subunit nuclear factor-kappa B essential modulator (NEMO). Upon cell stimulation the IKK complex phosphorylates the IκB proteins that keep the NF-κB transcription factors in the cytoplasm. This leads to ubiquitination and degradation of the IκB proteins. As a result the NF-κB transcription factors enter the nucleus and activate gene transcription. In the case of canonical NF-κB signaling, mainly RelA:p50 and c-Rel:p52 heterodimers translocate to the nucleus (Mercurio, Zhu et al. 1997; Woronicz, Gao et al. 1997; Yamaoka, Courtois et al. 1998).

The so called non-canonical NF-κB signaling pathway relies on activation through receptors that are mainly involved in developmental processes like the lymphotoxin beta receptor or B cell activating factor (BAFF). Here, IKK1 acts independent from IKK2 and NEMO (Senftleben, Cao et al. 2001; Derudder, Dejardin et al. 2003) and leads to the phosphorylation and proteolytic processing of p100 to p52 which leads to the nuclear accumulation of p52:RelB dimers (Senftleben, Cao et al. 2001).

Healthy hepatocytes are resistant to cytotoxic effects of TNF. NF-κB signaling may give an essential survival stimulus that balances the effects of the cell death machinery that are activated by TNF. If NF-κB signaling is impaired as reported in RelA, NEMO and IKK2 deficient mice, embryonic lethality results due to TNF induced degradation of the fetal liver (Beg, Sha et al. 1995; Li, Van Antwerp et al. 1999; Rudolph, Yeh et al. 2000). Additional deletion of TNF or TNFR I rescues the embryonic lethality of these mice (Doi, Marino et al. 1999). This *in vivo* evidence showing that NF-κB prevents TNF mediated cell death is also supported by *in vitro* studies with hepatocytes which, if transfected with a dominant negative IκBα, are sensitive to TNF induced apoptosis (Bellas, FitzGerald et al. 1997).

The activation of NF-κB influences the pathogenesis of several TNF-mediated hepatic diseases. In TNF induced liver injury, wild type mice are only sensitized to TNF induced cell death upon co-administration of D-Galactosamine (GalN). GalN is a hepatotoxin that blocks transcription specifically in hepatocytes, including the transcription of anti-apoptotic NF-κB target genes, and therefore sensitizes mice to TNF induced lethality (Decker and Keppler 1974). To the same extent GalN sensitizes mice to bacterial Lipopolysaccaride (LPS) a strong inducer of TNF. Moreover, in liver regeneration after partial hepatectomy, TNF induced NF-κB plays an important role due to the induction of interleukin 6 (IL-6) (Cressman,

Greenbaum et al. 1996). Liver NF-κB also seems to play a role in carcinogenesis. IKK2 liver deficient mice develop more hepatocellular carcinomas after diethylnitrosamine (DEN) treatment due to a higher rate of hepatocyte apoptosis and compensatory proliferation (Maeda, Kamata et al. 2005). Furthermore, in mice lacking NEMO specifically in the liver, cell death and the spontaneous development of chronic hepatitis result in the development of hepatocellular carcinomas. This process is initiated by hypersensitivity of NEMO deficient hepatocytes to oxidative stress dependent, FADD mediated apoptosis, triggering compensatory cell proliferation, inflammation and activation of progenitor cells (Luedde, Beraza et al. 2007). Similar to the DEN model, hepatocyte specific deletion of NEMO highlights the essential anti-apoptotic role of NF-κB avoiding cell death followed by compensatory proliferation and cancer. NF-κB also processes inflammatory responses since its activation does not only lead to the expression of anti-apoptotic mediators but also to the expression of inflammatory cytokines. Indeed, deletion of IKK2 in the liver seems to prevent liver insulin resistance due to a lower expression of inflammatory cytokines that affect insulin signaling (Arkan, Hevener et al. 2005) while liver specific hyperactivation of IKK2 has the opposite effect (Cai, Yuan et al. 2005).

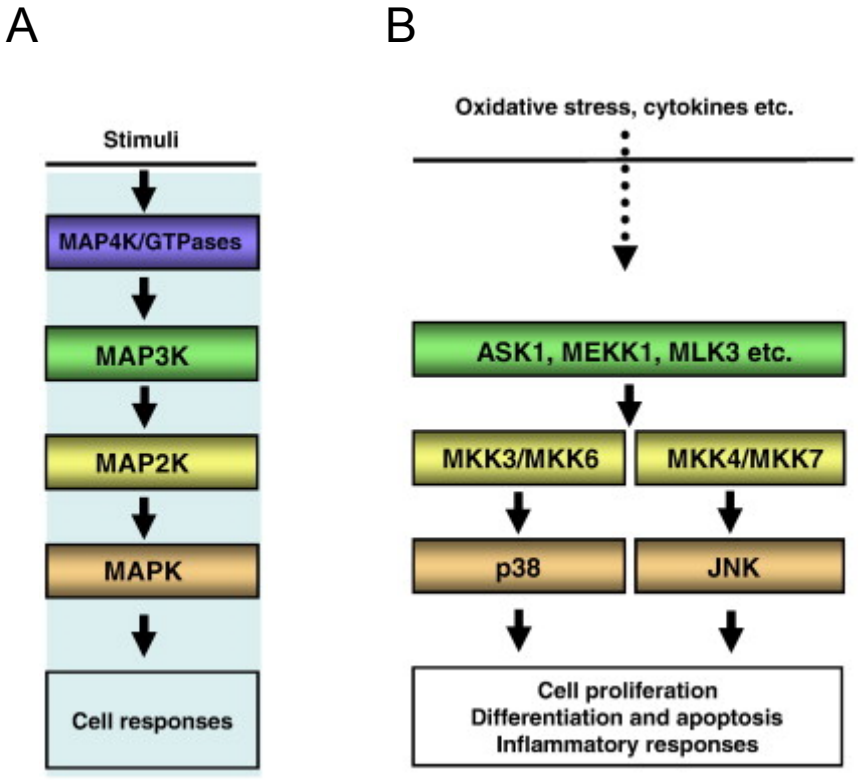
1.3.2 MAP kinase cascades

Besides NF-κB signaling, ligand binding to TNFR I also activates Mitogen-activated protein kinases (MAPKs), in particular c-Jun n-terminal kinases (JNK) and p38.

MAPKs are evolutionary conserved serine-threonine kinases that connect cell surface receptors to critical regulatory targets within the cells. Mammals express at least four distinctly related groups of MAPKs, (1) extracellular signal related kinases (ERK) 1/2, (2) Jun n-terminal kinases (JNK) 1/2/3, (3) p38 proteins α, β, γ, δ and (4) ERK 5.

MAPKs are activated by phosphorylation cascades. These cascades consist of a set of three evolutionarily conserved, sequentially acting kinases: (1) a MAPK, (2) a MAPK kinase (MAP2K), and (3) an MAPK kinase kinase (MAP3K). MAP3Ks, are Ser/Thr kinases that are activated via phosphorylation or through interaction with small GTP proteins of the Ras/Rho family in response to a wide range of extracellular stimuli. MAP3K activation leads to phosphorylation and activation of a downstream MAP2K. MAP2Ks are dual specificity kinases that can phosphorylate MAPK on both threonine and tyrosine on a conserved Thr-x-Tyr motif to activate them. The Thr-x-Tyr phosphorylation motif is localized in an activation loop near the ATP- and substrate-binding sites. Once activated, MAPKs phosphorylate their target substrates on serine or threonine residues only if the amino acid residues are followed

by proline. A wide variety of MAPK substrates exists, among them transcription factors, other kinases (MAPK activated kinases or MKs) or proteins like cytoskeletal proteins. Each of these cascades is named after the subgroup of its MAPK component ERK1/2, JNK, p38 or ERK 5. In mammals 20 genes encoding for MAP3Ks have been reported, followed by 7 MAP2Ks and 11 MAPKs genes (Uhlik, Abell et al. 2004). Different extracellular signals can produce stimulus- and tissue-specific responses by activating one or more MAPK pathways. In addition, MAPK-activated protein kinases (MKs), the downstream targets of MAPKs, further contribute to additional specificity, diversity and amplification in the MAPK cascades.



Graph 2: MAPK signaling. (A) MAPK signaling pathways mediate intracellular signaling that can be initiated by a variety of extracellular or intracellular stimuli. Initially, MAP3Ks, which are activated by MAP4Ks or GTPases, mediate phosphorylation and activation of MAP2Ks, which in turn phosphorylate and activate MAPKs. Activated MAPKs phosphorylate various substrate proteins including transcription factors, resulting in regulation of a variety of cellular activities including cell proliferation, differentiation, migration, inflammatory responses, and death. (B) The p38 and JNK pathways are activated by a MAP3K such as ASK1, MEKK1, or MLK3 as well as a MAP2K such as MKK3 or MKK6 for the p38 pathway and MKK4 or MKK7 for the JNK pathway. Pictures taken from Kyung, E *et al.* 2010 Biochimica et Biophysica Acta.

One of the most explored functions of MAPK signaling modules is the regulation of gene expression in response to extracellular stimuli (Treisman 1996). MAPKs function inside the nucleus and target transcription factors that are bound to DNA. It has also been reported that MAPKs regulate gene expression through post-transcriptional mechanisms involving cytoplasmic targets. (Chen, Gherzi et al. 2000).

The biological outcomes of MAPK signaling include a wide range of functions including cell proliferation, differentiation, survival, death and transformation. These functions are particularly important in an organ like the liver which has a high proliferative and regenerative capacity and always balances between cell survival and cell death. In contrast to NF-κB, MAPK signaling is considered to be rather diverse with pro-apoptotic but also anti-apoptotic functions.

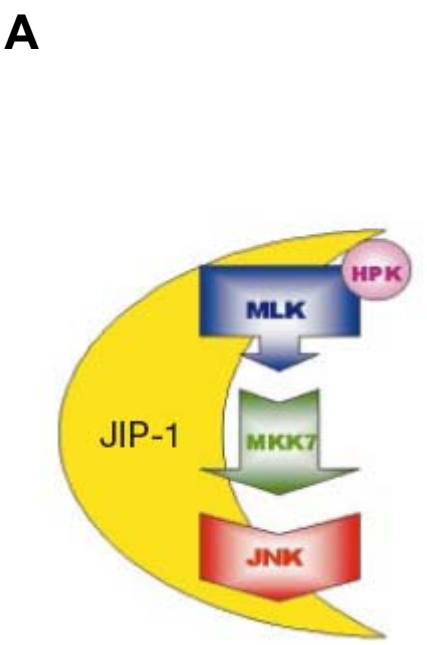
Two members of the MAPK family, JNK and the p38 signaling pathway, can be activated by TNF in response to cellular stress such as genotoxic, osmotic, hypoxic or oxidative stress.

1.3.3 JNK signaling in the liver

The family of JNK protein kinases is encoded by 3 different genes. The JNK 1 and JNK 2 genes are expressed ubiquitously while JNK 3 expression has a limited pattern and is restricted to the brain, heart and testis (Gupta, Barrett et al. 1996).

JNK is activated by the dual specificity MAP2Ks MKK4 and MKK7, which are activated by their upstream MAP3Ks through phosphorylation on serine or threonine residues in the activation loop. A wide range of MAP3Ks have neen described, which most likely act in a cell type and stimulus-specific manner (Davis 1995). The first and most potent JNK-activating MAP3K identified was MEKK1 (Gerwins, Blank et al. 1997). However, numerous other MAP3Ks, which can activate JNK were identified, including: MEKK2 and MEKK3 (Blank, Gerwins et al. 1996), MEKK4 (Gerwins, Blank et al. 1997), Mixed Lineage kinases 2 and 3 (MLK2 and MLK3) (Hirai, Izawa et al. 1996; Tibbles, Ing et al. 1996), Dual leucine zipper-bearing kinase (DLK) (Fan, Merritt et al. 1996), Tumor progression locus-2 (Tpl-2) (Salmeron, Ahmad et al. 1996), TGF-β-activated kinase (TAK1) (Yamaguchi, Shirakabe et al. 1995), Apoptosis Signal Regulating Kinases 1 and 2 (ASK1 and ASK2) (Ichijo, Nishida et al. 1997), and Thousand and one amino acid kinases 1 and 2 (Tao1 and Tao2) (Chen, Hutchison et al. 1999). The large number of MAP3Ks that can activate the JNK cascade have made the identification of the individual family member responsible for JNK activation by distinct stimuli extremely difficult.
The organization of MAPK cascades as distinct signaling modules allows their highly coordinated and specific activation response to extracellular stimuli (Davis 2000). Thus, the presence of a large number of JNK-activating MAP3Ks in mammals may provide the benefit of improved signal specificity to diverse types of physiological and stress stimuli.

Another level of regulation adding specificity to JNK signaling is the scaffolding of proteins, which includes the JNK Interacting Proteins (JIP) like scaffolds (Davis 1999) and the MKKs. (Xia, Wu et al. 1998; Morrison and Davis 2003). Besides the purpose of specificity, scaffolding might also serve the purpose of amplifying a signal.



Graph 3: JNK Scaffolding JIP-1 can act as a scaffold assembling specific components of JNK activation including the upstream kinase HPK-1, the MAP3K MLK1, the MA2K MKK7 and the MAPK JNK into a signaling module. Picture taken from Chang L *et al.* 2001 Nature.

JNK proteins phosphorylate a wide range of transcription factors including c-Jun which is phosphorylated at Ser 63 and 74 leading to increased transcriptional activity (Pulverer, Kyriakis et al. 1991) and AP-1 proteins like JunB, JunD and Atf2 (Auer, Contessa et al. 1998; Ip and Davis 1998). JNK appears to be important for AP-1 target gene activation caused by stress and cytokines but is not required for AP-1 transcriptional activation by other stimuli (Yang, Tournier et al. 1997).

In recent years, JNK isoforms have been recognized as critical regulators of various aspects of mammalian physiology, including: cell proliferation, cell survival, cell death, DNA repair and metabolism. Upon TNF signaling, the duration and magnitude of JNK activation seems to be determined by the balance between activating MKKs. Only a modest and transient JNK activation is required for TNF-induced cell proliferation, whereas an intense and prolonged JNK activation mediates cell death (Kamata, Honda et al. 2005) highlighting the critical balance of TNF induced JNK activation for the outcome of the biological response.

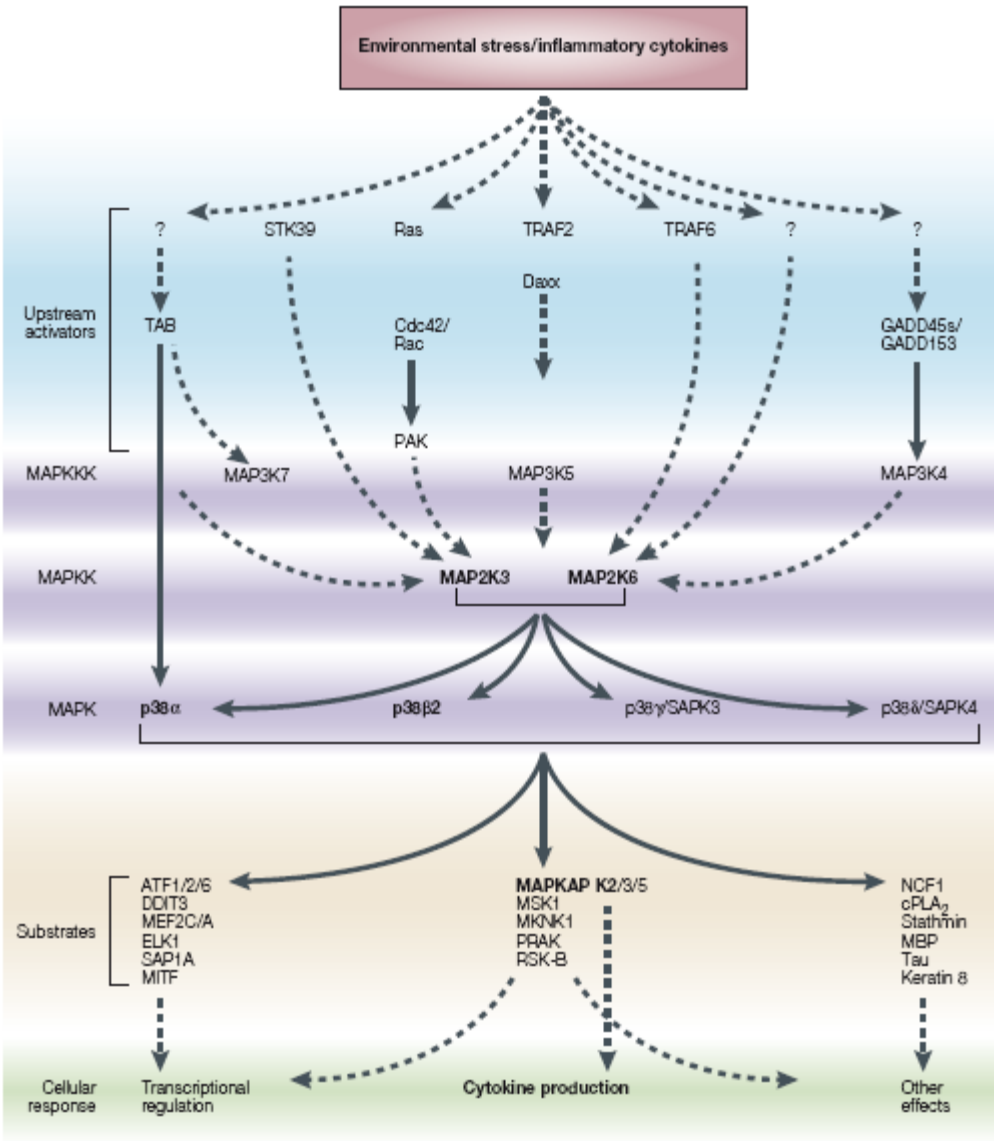
After partial hepatectomy, which induces TNF, JNK is activated and is instrumental for liver regeneration (Schwabe, Bradham et al. 2003). On the other hand, in livers and hepatocytes unable to activate NF-κB, JNK activity controls TNF-induced death through the proteasomal processing of c-FLIP (L) (Chang, Kamata et al. 2006). Moreover, JNK plays a role in DEN induced hepatocarcinogenesis. In hepatocytes, NF-κB activation promotes sustained JNK activation in cells exposed to TNF, whose expression is induced by DEN. JNK activity is required for normal hepatocyte proliferation and liver regeneration. Mice lacking JNK1 were much less susceptible to DEN-induced hepatocarcinogenesis. The decreased tumorigenesis in $JNK1^{-/-}$ mice was shown to correlate with decreased expression of cyclin D and vascular endothelial growth factor, diminished cell proliferation, and reduced tumor neovascularization (Sakurai, Maeda et al. 2006). JNK also seems to be involved in the negative regulation of insulin signaling. It has been demonstrated *in vitro* that JNKs can phosphorylate the insulin receptor substrate (IRS1) on serine 307, which correlates with reduced Insulin receptor mediated IRS1 tyrosine phosphorylation leading to a decreased transduction of the insulin stimulus (Aguirre, Uchida et al. 2000). Moreover, JNK isoforms undergo activation in response to pro inflammatory cytokines, such as TNF, which is elevated during obesity (Kallunki, Su et al. 1994). Indeed, it was observed that consumption of high fat diet (HFD) or genetic obesity results in JNK activation (Hirosumi, Tuncman et al. 2002) and that $JNK1^{-/-}$ mice are protected from diet induced obesity and insulin resistance, even though in this model the tissue specific contributions of JNK1 to this phenotype were not addressed. Furthermore, treatment of obese mice with a cell permeable peptide inhibitor of JNK was found to result in improved insulin sensitivity (Bogoyevitch, Boehm et al. 2004). It is also of interest that mutations in the Jip1 gene have been found in patients with a genetic form of type II diabetes (Waeber, Delplanque et al. 2000). This suggests that a JIP1-organized JNK1 cascade plays an important role in the regulation of insulin signaling.

In contrast to JNK, which has been studied in various different models in the liver, the functions of p38, another MAPK activated by TNF signaling, are far less understood.

1.3.4 p38 MAPK

Human p38 was originally discovered as the molecular target of the pyridinyl imidazole class of compounds that were known to inhibit biosynthesis of inflammatory cytokines such as interleukin-1 (IL-1) and TNF in lipopolysaccharide (LPS) stimulated human monocytes (Lee, Laydon et al. 1994). Later, the murine p38α MAPK was identified as a major phosphoprotein activated by LPS and inflammatory cytokines (Han, Lee et al. 1994). So far, four isoforms of p38 MAPK have been identified. Of these, the ubiquitously expressed p38α is the best characterized and perhaps the most physiologically relevant kinase involved in inflammatory responses. Besides p38α, p38β was identified which has >70% identity to p38α (Jiang, Chen et al. 1996; Kumar, McDonnell et al. 1997). Later, p38γ (also known as SAPK3 and MAPK12) and p38δ (also known as SAPK4 and MAPK13) (Li, Jiang et al. 1996; Mertens, Craxton et al. 1996) were identified. These kinases have ~60% identity to p38α and much less is known about their function.

p38 MAPK is activated though a classical MAPK activation cascade involving a MAP3K, that activates a MAP2K which then activates the MAPK p38. Activation of p38 occurs by dual phosphorylation on Thr180 and Tyr182 by an upstream MAP2K termed MKK6. Other MAP2Ks, such as MKK3 have also been suggested to activate p38 MAPK. MKK6 is activated by several MAP3Ks, which are in turn activated by a wide variety of stimuli including MAP3Ks activated in response to various physical and chemical stresses, such as oxidative stress, UV irradiation, hypoxia, ischemia, and various cytokines, including interleukin-1 (IL-1) and TNF (Adams, Badger et al. 2001; Chen, Gibson et al. 2001; Kyriakis and Avruch 2001).



Graph 4: p38 MAPK pathway activators and substrates Environmental stress and/or inflammatory cytokines activate MAP3Ks through a variety of signaling mechanisms. Activated MAP3Ks subsequently activate MAP2Ks, which in turn activate MAPKs. Activated MAPKs then phosphorylate several substrates, such as transcription factors, other kinases and cytosolic proteins. Picture taken from Kumar *et al.* 2003 Nat. Rev.

MEK4 (also known as MKK4 and Sek1) is a known JNK kinase that possesses some MAP2K activity toward p38, suggesting that MEK4 represents a site of integration for the p38 and JNK pathways. While MKK6 activates all p38 isoforms, MKK3 is somewhat selective, as it preferentially phosphorylates the p38α and p38β isoforms.

The first p38α substrate identified was the MAP kinase- activated protein kinase 2 (MAPKAPK2 or MK2) (Freshney, Rawlinson et al. 1994; Rouse, Cohen et al. 1994; McLaughlin, Kumar et al. 1996). MK2, along with its closely related family member MK3,

were both shown to activate various substrates including small heat shock protein 27 (HSP27) (Stokoe, Engel et al. 1992), lymphocyte-specific protein 1 (LSP1) (Huang, Zhan et al. 1997), cAMP response element-binding protein (CREB) (Tan, Rouse et al. 1996), transcription factor ATF1, SRF (Heidenreich, Neininger et al. 1999), and tyrosine hydroxylase (Thomas, Haavik et al. 1997). MNK1 is another kinase substrate of p38 whose function is thought to reside in translational initiation due to the observation that MNK1 and MNK2 can phosphorylate eukaryotic initiation factor-4e (eIF-4E) (Waskiewicz, Flynn et al. 1997).
Another group of substrates that are activated by p38 comprises transcription factors. Many transcription factors encompassing a broad range of action have been shown to be phosphorylated and subsequently activated by p38. Examples include activating transcription factor 1, 2 & 6 (ATF-1/2/6), SRF accessory protein (Sap1), CHOP (growth arrest and DNA damage inducible gene 153, or GADD153), p53, C/EBPβ, myocyte enhance factor 2C (MEF2C), MEF2A, MITF1, DDIT3, ELK1, NFAT, and high mobility group-box protein 1 (HBP1) (Hazzalin, Cano et al. 1996; Wang and Ron 1996; Han, Jiang et al. 1997; Janknecht and Hunter 1997; Whitmarsh, Yang et al. 1997; Huang, Ma et al. 1999; Zhao, New et al. 1999; Yee, Paulson et al. 2004). An important *cis*-element, AP-1 appears to be influenced by p38 through several different mechanisms. ATF-2 is known to form heterodimers with Jun family transcription factors thereby directly associating with the AP- 1 binding site. Another possible mechanism comes from the observation that a component of AP-1 is c-fos. c-fos is known to be SRE dependent and SRE is able to bind Ternary Complex Factor (TCF). Ternary Complex mFactor is comprised of Sap-1a, a protein that is phosphorylated by p38. Thus, p38 indirectly regulates AP-1 activity.

Considering the wide range of substrates linked to p38 MAPKs, it is a natural consequence that this kinase should also be involved in a wide and diverse variety of functions. Indeed, roles for p38 MAPKs have been described in processes such as cell differentiation and migration, cell transformation, cell survival, inflammation, angiogenesis, myogenesis and others.

The fact that $p38^{-/-}$ mice are lethal at embryonic day 10.5 has deprived researchers of an important genetic tool for functional studies *in vivo*. Four groups have reported the generation of these mice (Adams, Porras et al. 2000; Allen, Svensson et al. 2000; Mudgett, Ding et al. 2000; Tamura, Sudo et al. 2000). Insufficient vascularization during placental development was described as a possible cause of death. Mudget et al. found that in addition to the placental defect, angiogenesis was also abnormal in the yolk sac and the embryo itself. Adams et al. reported massive reduction of the myocardium and malformation of blood vessels in the head region. However, this defect appears to be secondary to insufficient

oxygen and nutrient transfer across the placenta. Finally, fetuses surviving to relatively late stages of development are anemic and have a deficiency of erythropoietin (Epo) mRNA expression in their liver. Therefore, the studies conducted on p38 function have been restricted to *in vitro* assays and stimulus specific functions in the context of a specific organ like the liver could not be addressed so far.

Among other processes, p38 seems to play a role in inflammation. Rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease are all postulated to be regulated in part by the p38 pathway (Perregaux, Dean et al. 1995; Johnson and Bailey 2003; Hollenbach, Neumann et al. 2004). p38 signaling seems to play an essential role in the production of proinflammatory cytokines (IL-1β, TNF and IL-6) (Guan, Buckman et al. 1998). Moreover, p38 has a role in the expression of intracellular enzymes such as iNOS, a regulator of oxidation (Da Silva, Pierrat et al. 1997; Craxton, Shu et al. 1998), induction of VCAM-1 and other adherent proteins along with other inflammatory related molecules (Pietersma, Tilly et al. 1997).

The role of p38 in apoptosis seems to be cell type and stimulus dependent. While p38 signaling has been shown to promote cell death in some cell lines, in other cell lines p38 has been shown to enhance survival, cell growth, and differentiation. There is evidence that p38 might act up and downstream of caspases in apoptosis (Ziegler-Heitbrock, Blumenstein et al. 1992; Cardone, Salvesen et al. 1997).

Furthermore, the participation of p38 in cell growth has been observed in yeast and mammals (Takenaka, Moriguchi et al. 1998). Overexpression of p38 in yeast led to significant attenuation of proliferation, while treatment in mammalian cells with p38α/β inhibitor SB203580 slowed proliferation as well. p38 has been implicated in G1 and G2/M phases of the cell cycle in several reports (Molnar, Theodoras et al. 1997; Wang, McGowan et al. 2000).

p38α and p38β have been implicated in the differentiation of certain cell types. Differentiation of 3T3-L1 cells into adipocytes and PC12 cells into neurons requires p38α and/or β (Engelman, Lisanti et al. 1998). More recently, a cross-talk model between the p38 pathway and phosphatidylinositol 3-kinase (PI3 kinase)/Akt in the orchestration of myoblast differentiation has been proposed (Iwasa, Han et al. 2003).

Moreover, p38 seems to have a role in tumorigenesis and sensescence. There have been reports that activation of MKK6 and MKK3 led to a senescent phenotype dependent upon p38 MAPK activity. Also, p38 MAPK activity was shown responsible for senescence in response to telomere shortening, H_2O_2 exposure, and chronic RAS oncogene signaling (Wang, Chen et al. 2002).

p38 might also be involved in the energy metabolism of certain organs. Here, most studies are restricted to *in vitro* work. The few *in vivo studies* were carried out with p38 inhibitors. In

the liver, p38 might participate in the processes to increase blood glucose levels through reducing glycogen synthesis and increasing hepatic gluconeogenesis (Cao, Collins et al. 2005). p38 appears to prevent fat storage by inhibiting hepatic lipogenesis and promoting fatty acid oxidation in the liver. Additionally, p38 may play a critical role in cholesterol metabolism by regulating expression of the LDLR gene and bile metabolism, but many functions remain controversial (Cao, Collins et al. 2005; Cao, Xiong et al. 2007).

1.4 The role of p38α in acute and chronic inflammatory diseases

As mentioned before, a strong link has been established between the p38α pathway and inflammation. Rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease are all postulated to be regulated in part by the p38 signaling pathway (Johnson and Bailey 2003; Hollenbach, Neumann et al. 2004). Furthermore, the activation of the p38 pathway plays an essential role in the production of proinflammatory cytokines like IL-1β, TNF and IL-6 (Guan, Buckman et al. 1998). All three cytokines were reported to play important roles in liver homeostasis and disease. In contrast to NF-κB and JNK, the tissue specific role of liver p38α during various inflammatory conditions remains to be investigated *in vivo*. Thus, as a central topic of this thesis, the role of liver p38α in acute and chronic inflammatory reactions was investigated. In order to do this, two mouse models of inflammation were chosen. For the role of liver p38α in acute inflammatory reactions, a model of LPS/TNF induced acute liver toxicity was applied. To investigate the role of p38α in chronic low grade inflammatory reactions a model of diet induced obesity and insulin resistance was utilized.

1.4.1 LPS/TNF induced acute liver failure

The liver is the central metabolic organ in the body fulfilling important functions ranging from carbohydrate and lipid metabolism, to synthetic and excretory functions. Due to these central functions processed in the liver, diseases of this organ can lead to severe symptoms. The liver has an outstanding potential to regenerate and is able to compensate lack of function for an extended period of time. Therefore, symptoms arise very late during the course of disease, which makes diagnosis and therapy of liver diseases extremely difficult. Often, liver damage is recognized at a very late stage (Sass and Shakil 2003).

Acute liver damage can be caused by a wide range of substances, including toxins and drugs like Paracetamol or Halothan. In addition, sepsis or ischemic hepatitis after surgery can lead to acute liver failure. The liver is able to tolerate and compensate chronic sickness like viral infections, parasites or metabolic disorders to a certain degree. Nevertheless, once

the amount of intact hepatocytes falls below a certain threshold, acute liver failure is the consequence.

Two molecular mechanisms were identified to cause liver failure: (1) Necrosis. Due to disruption of cellular metabolism caused by various insults, acute lack of ATP and dysregulation of ion homeostasis lead to cell swelling and rupture of the cell membrane. As a result intracellular proteins, ions and metabolites leak into the surrounding tissue causing recruitment of immune cells and inflammatory reactions (Bauer 2002). (2) The second molecular mechanism that can induce liver failure is programmed cell death (Apoptosis). Apoptosis is an essential process during embryonic development and tissue remodeling. Cells die without affecting the surrounding tissue since they are phagocytozed by immune cells. Intracellular pathways like the caspase cascades lead to rapid apoptosis of cells (Neuman 2001). In the liver, apoptosis is initiated during viral infections or by inflammatory cytokines like TNF when necessary survival stimuli are not present. TNF seems to have an outstanding role in liver failure caused by ischemia/reperfusion injury (Colletti, Remick et al. 1990), alcohol induced hepatitis (Felver, Mezey et al. 1990), chronic viral hepatitis (Gonzalez-Amaro, Garcia-Monzon et al. 1994) and poisoning by toxins (Leist, Gantner et al. 1997). Elevated serum levels of TNF were shown in patients with liver failure (Muto, Nouri-Aria et al. 1988). Patients that died from acute liver failure showed significantly higher levels of TNF in their serum than patients that survived (Bird, Sheron et al. 1990). The amount of apoptotic or necrotic hepatocytes can be directly correlated to the expression of TNF and underlines the outstanding role TNF has during the development of liver failure (Streetz, Leifeld et al. 2000). In light of the importance that TNF has in the development of acute liver failure it is essential to understand the molecular mechanisms that underlie this process. TNF is known to activate MAP kinases including p38α. In order to find new targets for medical intervention in cases of acute liver failure it is important to gain more knowledge about the molecular events that precede this severe medical condition.

1.4.2 Chronic inflammatory states in the liver: diet induced obesity and insulin resistance

The incidence of obesity worldwide has increased drastically during recent decades. Consequently, obesity and associated disorders now constitute a serious threat to the current and future health of the world population. The World Health Organization estimates that more than 1 billion adults worldwide are overweight, 300 million of who are clinically obese (World Health Organization, 2002 Geneva). With obesity, comes a variety of adverse health outcomes, such as high blood pressure, insulin resistance, and type 2 diabetes (Mokdad, Ford et al. 2003; Reaven 2005).

The metabolic disorder diabetes mellitus is characterized by chronic hyperglycemia, caused by an absolute or relative insulin deficiency. Continuously high blood sugar levels interfere with numerous metabolic processes and, as a consequence, lead to severe tissue damage.
Only a minority of patients suffers from an absolute insulin deficiency. This so-called Type 1 diabetes is caused by the complete absence of insulin secondary to autoimmune destruction of the insulin-producing islet β-cells in the pancreas (Eisenbarth, Nayak et al. 1988). In the vast majority of patients, diabetes is triggered by a combination of genetic and environmental factors, which interfere with the body's ability to react to insulin (type 2 diabetes).

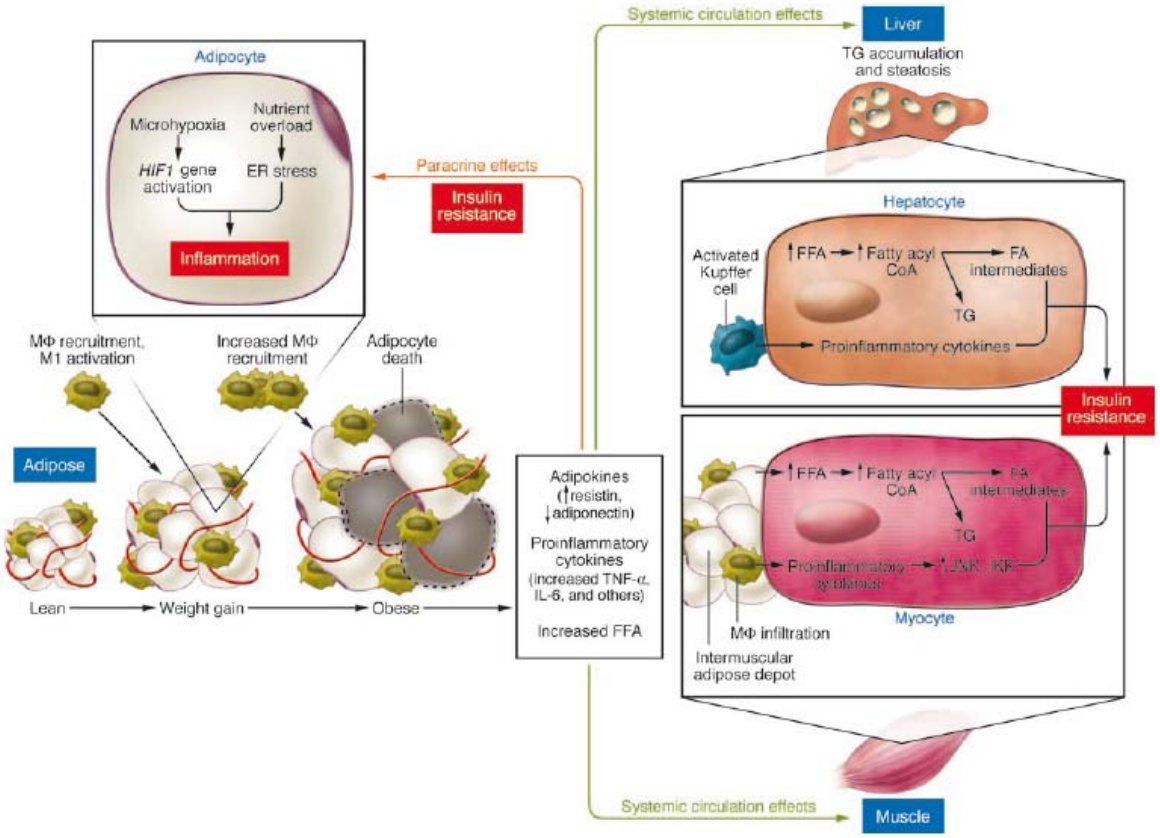
Insulin resistance, a hallmark of Type 2 diabetes is defined as an inadequate response of insulin target tissues, such as skeletal muscle, liver, and adipose tissue, to the physiologic effects of circulating insulin. The hallmarks of impaired insulin sensitivity in these three tissues are decreased insulin-stimulated glucose uptake into skeletal muscle, impaired insulin-mediated inhibition of hepatic glucose production in the liver, and a reduced ability of insulin to inhibit lipolysis in adipose tissue. Pancreatic β-cells counteract insulin resistance by increasing the production and release of insulin resulting in a compensatory hyperinsulinemia (Perley and Kipnis 1966; Polonsky, Given et al. 1988; Kahn, Prigeon et al. 1993). With time, the progressive deterioration in β-cell function contributes to the characteristic relative insulin deficiency and aggravates hyperglycemia (DeFronzo 1997).

Type 2 diabetes is a well characterized disease and has been known for many years. Nevertheless, the molecular mechanisms underlying the development of insulin resistance are still not fully understood. During the past decade, it became clear that inflammation is a key feature of obesity and Type 2 diabetes (Wellen and Hotamisligil 2005). Currently, there are two main theories that explain the molecular mechanisms underlying the development of insulin resistance.

1.Inflammation. Weight gain during the course of obesity induces low grade chronic inflammation in white adipose tissue leading to local insulin resistance and the release of pro-inflammatory cytokines such as TNF and IL-6 into the circulation (Hotamisligil, Shargill et al. 1993). Inflammatory cytokines inhibit insulin action in classical insulin target tissues such as skeletal muscle, liver and adipose cells, thereby resulting in impaired glucose homeostasis (Hotamisligil, Budavari et al. 1994; Plomgaard, Bouzakri et al. 2005).

2.Lipotoxicity. Insulin resistance in the adipose tissue leads to elevated release of lipids into the circulation. Once the capacity of the skeletal muscle and liver to dispose fatty acids via oxidation or storage is exceeded, various fatty acid intermediates, including Diacylglycerols and Ceramides, accumulate in skeletal muscle and liver and negatively regulate insulin

action (Bachmann, Dahl et al. 2001; Morino, Petersen et al. 2006; Summers 2006). Nutrient sensors like p70S6K and the PKC pathway are activated and lead to an impairment of insulin signaling in concert with a subsequent activation of inflammatory signaling pathways like JNK and NF-kB (Sathyanarayana, Barthwal et al. 2002; Jean-Baptiste, Yang et al. 2005; Sampson and Cooper 2006; Wang, Devaiah et al. 2006).



Graph 5: Obesity, tissue inflammation, and insulin resistance Picture taken from Olefsky *et al.* JCI 2008

In summary, inflammatory cytokines including TNF and lipids seem to play an important role initiating a chronic low grade inflammatory state in peripheral insulin sensitive organs. Among the insulin sensitive tissues, the liver is of central importance for glucose homeostasis. It is able to store excess glucose after food intake in the form of glycogen and releases glucose produced by glycogenolysis or gluconeogenesis during fasting in order to maintain steady blood glucose levels (Gribble 2005). Moreover, the liver is able to inhibit glycogenolysis and gluconeogenesis by controlling gene expression or activity of rate limiting enzymes. Dysregulation of insulin action in the liver is a key component in the pathogenesis of Type 2 diabetes. (Saltiel and Kahn 2001). Therefore, it is important to understand the molecular basis of chronic inflammatory reactions and the mechanisms that impair insulin signaling, especially in the liver.

1.5 The cre/ loxP system

As described before, p38α$^{-/-}$ mice are embryonic lethal. To investigate the role of p38α in the liver a conditional mouse model had to be utilized which leads to the deletion of p38α after embryonic development. Therefore, mice with a liver parenchymal cell specific deletion of p38α were generated using the Cre/LoxP system. The Cre/LoxP system is derived from the bacteriophage P1 and allows site specific recombination of DNA strands. The system consists of an enzyme (Cre) that catalyses recombination of DNA between specific sites (LoxP sites). A loxP site is 34 base pairs long and consists of an asymmetric 8 base pair sequence that is flanked by 13 base pair palindromic sequences on both sides. These palindromic sequences act as binding sites for the Cre recombinase. The recombination then occurs in the asymmetric sequence. As a result a reciprocal recombination event may take place between two LoxP sites if cre is expressed in a cell. The asymmetric sequence gives directionality to the LoxP site. Important for the outcome of the recombination is the spatial orientation of two LoxP sites one against the other. If two sites are placed on the same arm of the chromosome and have the same direction, the recombination will result in a deletion of the DNA region that was located between the LoxP sites. This effect is commonly used in genetics in order to introduce mutations or delete gene regions in a controlled and inducible manner. The loxP sites are placed at the desired location of the genome by homologous recombination. The presence of the mutation or deletion is regulated via transducing Cre into cells or via expressing Cre under the control of an inducible or tissue specific promoter.

In order to delete p38α specifically in hepatocytes p38α^{FL} mice were crossed with mice carrying the Alfp cre transgene (Reichardt, Kellendonk et al. 1999). The p38α^{FL} mice carry loxP sites flanking exons 2 and 3, which include the ATP-binding site of the kinase domain.

1.6 Objectives

In terms of its cellular composition the liver is a heterogeneous tissue and cytokines are key messengers orchestrating the numerous important functions to keep the body homeostasis. TNF is an important molecule in this complex interplay of different cell types. Therefore, a healthy balance of TNF induced signaling is crucial to maintain liver functions. Dysregulated cytokine signaling can shift biological responses in an unfavorable direction. This can lead to serious impairment in liver functions having an impact on whole body metabolism or lead to liver failure. Understanding the functional complexity of the downstream pathways activated by TNF signaling could potentially identify novel targets for the treatment of many acute and chronic liver diseases.

Important progress has been made in order to gain some understanding of the functions that the signaling cascades activated by TNFR I signaling process. Many functions of the NF-κB signaling pathway and JNK MAPK cascade in the liver have been investigated in detail. The functions of p38, another MAPK activated upon TNF signaling, are less understood. This is partly due to the fact that $p38\alpha^{-/-}$ mice are embryonic lethal, preventing extensive *in vivo* studies.

Making use of conditional gene targeting, the aim of the thesis was to study the role p38α in the liver during acute and chronic inflammatory states, *in vivo*. Due to the outstanding role of TNF in tissue homeostasis, two models that induce significantly elevated levels of circulating TNF were chosen to study the role of p38α in different inflammatory conditions in the liver.

To study the role of the MAPK p38 during the development of acute liver failure, a model of bacterial Lipopolyscaacride (LPS) was applied. LPS is one of the most potent TNF inducers but usually does not cause hepatocyte apoptosis unless it is co-administered with D-Galactasoamine (GalN) which specifically blocks transcription in the liver (Decker and Keppler 1974; Bradham, Plumpe et al. 1998). The aim of the first part of this thesis was to analyze the LPS/TNF induced apoptotic signals in mice specifically lacking p38α in liver parenchymal cells.

In order to study the role in chronic low grade inflammatory states, a model of high fat diet induced obesity and insulin resistance was utilized. Type 2 diabetes is a complex multifactorial disease triggered by the combination of β- cell dysfunction and insulin resistance of target tissues. Insulin resistance results from impaired activation of the enzymatic cascade of phosphorylation events in the insulin signaling pathway. Although a huge variety of processes involved in the development of insulin resistance has already been

described, the underlying exact molecular mechanisms are still to be investigated. Therefore the objective of the second part of this thesis was to analyze the role of liver p38α in chronic sub-acute inflammatory states caused by high fat diet feeding and its influence on glucose homeostasis and insulin sensitivity.

2 Material and Methods

2.1 Material

Solutions:

Hepatocyte culture medium: High Glucose DEMEM, 5% FCS, Pen/Strep.

Ketamin-Rompun solution: 5ml Ketamin (10mg/ml), 230µl 2% Rompun.

Loading Dye DNA: 15% Ficoll 400 in ddH_2O, heat to 50 °C then add Organe G ad libidum, autoclave.

Loading Dye Protein 5x (10ml): 1,1ml Tris HCL pH 6,8, 2ml Glycerol, 3,8ml 10% SDS, 800µl ß-mercaptoethanol.

NP-40 Lysis Buffer (500ml): 452,5ml ddH_2O, 25ml 1M Tris HCL pH 7,5, 15ml 5M NaCL, 2,5ml NP-40, 1,05g NaF. Before use add 1 tablet Protease and Phospahtase inhibitor to 10 ml of buffer.

Running buffer (1l): 30,25g Tris Base, 144,2g Glycine, 10g SDS. pH was not adjusted.

Starving medium: High Glucose DEMEM, Pen/Strep

TAE Buffer 25x (10l): 1210g Tris, 0.5l EDTA pH 8,0 500 m, 285,5mL Acetic acid

Tail Lysis Buffer (1l): 100ml Tris HCL pH 8,5, 20ml 10% SDS, 11,68 NaCL, 1,86g EDTA.

TBS (10x) 1l: 24,2g Tris Base, 80g NaCl. Adjust pH to 7,6 with 37% HCL

TBS/T: add 0,1% of Tween to TBS

Transfer Buffer (1l): 3g Tris Base, 14,3g Glycine, 20% Methanol. Do not adjust pH.

Oil Red O Solutin: 1,5g Oil Red O dissolved in 300ml Isopropanol over night, then 200ml of ddH_2O are added.

RIPA buffer high salt (50ml): 1ml 1M HEPES, 3,5ml 5M NaCL, 10ml Glycerol, 50µl 1M $MgCL_2$, 50µl 500mM EDTA, 50µl 0,1M EGTA, add ddH_2O to 50ml. Before lysis add 1 tablet Protease and Phospahtase inhibitor to 10 ml of buffer, 1% NP-40, 0,1mM NaF.

Material

Acrylamide: BioRad 30% Acrylamide/Bis solution Ref: 161-0158

Agarose: BioZym Ref: 840004

Amonium Persulfate: Sigma, Ref:

Bornyle: Adsyte Pro, 22 GA 0,9 x 25mm, BD, Ref: 388714

Chloroform: Applichem

Collagenase: Collagenase Type II, Worthington, Ref: 4176 Lot: 47D9570

Culture dish (Collagen coated) 10cm:

DMEM: Gibco: 4,5g/l glucose, Ref: 41965

DNA Ladder: PeqLab, Ref: 25-2000

DNase: Qiagen RNase free DNase set Ref: 79254

EBSS -CA, -MG: Gibco, Ref: 14155

EBSS +CA, +MG: Gibco, Ref: 24010

ECL solution: GE Healthcare, Ref: PRN2209

ECL plus: GE Healthcare, Ref: PRN2132

EGTA: AppliChem

Ficoll 400: AppliChem A4969,0100, 100g

Films: Amersham Hyperfilm ECL, Ref: 28906837

Genotyping Primer :

Alfp cre: FW: 5'- TCC AGA TGG CAA ACA TAC GC- 3' REV: 5' – GTG TAC GGT CAG TAA ATT GGA C- 3'

p38α: FW: 5' CTACAGAATGCACCTCGGATG 3' REV: 5' AGAAGGCTGGATTTGCACAAG 3' REV: 5' CCAGCACTTGGAAGGCTATTC 3'

IKK2: FW: 5' - GTT CAG AGG TTC AGT CCA TTA TC - 3' REV: 5' - TAG CCT GCA AGA GAC AAT ACG - 3' REV: 5' - TCC TCT CCT CGT CAT CCT TCG - 3'

HEPES 1M: Gibco, Ref: 15630-056

Insulin: Insuman Rapid, 40 I.E./ml Sanofi Aventis

Insulin assay kit (serum): Chrystal Chem, Ref: INSKR020

Ketamin: Ketamin-Ratiopharm 50mg (10mg/ml)

LPS: Sigma

NaCl: Sigma

Nylon mesh: Falcon Cell strainer, BD, Ref: 352350

Oil Red O: AppliChem

Orange G: AppliChem A1404,0025 25 g

Penceau S: ApplicChem Ref: A2935

Protein Marker: PeqGold Protein Marker V, PeqLab, Ref: 27-2210

Phospatase inhibitor: PhosStop, Roche, Ref: 04 906 837 001

Protease Inhibitor: Complete Mini, EDTA free, Roche, Ref: 04 693 159 001

PVDF membrane: Immobilon P, Millipore, Ref: IPVH00010

Rompun 2%: Bayer Healthcare

RNA isilation Kit: Qiagen, RNeasy Mini, Ref: 74104

RT kit: SuperScript III Invitrogen Ref: 18080-051

SDS: AppliChem

Taquman Master mix:

Applied Biosystems, Taqman Gene Expression Master Mix, Ref: 4369016

Taquman Probes:

TNF: Mn00443258_m1

MCP-1: Mn00441242_m1

IL-6: Mn00446190_m1

IL1β: Mn00434228_m1

Gapdh: Mn99999915_g1

TEMED: Merk

Trizol: Invitrogen, Ref: 15596-018

Trypan Blue 0,4%: Sigma, Ref: T8154

Trypsin inhibitor: Sigma T9128

Tween: Tween 20, Sigma-Aldrich Ref: P1379

Tunnel Kit: DeadEnd Fluorometric TUNEL System, Promega, Ref: 63250

Tris: Sigma

Western Blot stripping buffer: Thermo scientiffic, Ref: 21059

Tools/machines:

Gel chamber WB: BioRad

Glucomen: GlucoMen Visio, A.Menarini Ref: Ref: 36364

Glucomen Test stripes: A.Menarini Ref: 36364

Homogenizer: Precellys 24, PeqLab Ref: 91-PCS24

Nanodrop: PeqLab Ref: 91-ND-2000

Pump: Masterflex Easy load II, Cole&Parmer, Model: 77202-50

RT machine: 7900 Fast Real Time PCR System Applied Bilsosystems

Clinical analyzer: Cobas 1001 (Roche)

2.2 Methods

2.2.1 Molecular biology

2.2.1.1 Preparation of genomic DNA from tail biopsies of mutant mice

Tails biopsies were lysed in tail lysis buffer rotating at 56 °C for minimum 2 hours. The cell debris was spun down for 5 min at 14000rpm and the supernatant was mixed 1:1 with isopropanol to extract the DNA. The DNA was precipitated by centrifuging for 5 min at 14000rpm. After discarding the supernatant the pellet was washed with 500µl of 70% Ethanol were added. The samples were spun at 14000rpm for 5 minutes, air dried for 10 minutes at room temperature and resuspended in 100µl ddH_2O. 2µl of DNA were used for each PCR reaction.

2.2.1.2 Polymerase chain rection (PCR)

The polymerase chain reaction (PCR) was used for amplification of DNA fragments *in vitro* for mouse genotyping (Saiki, Scharf et al. 1985). Genotyping of mice was performed in a total reaction volume of 25 µl containing a maximum of 500 ng template DNA isolated from tail biopsies, 25 pmol of each primer.

2.2.1.3 Agarose gel electrophoresis and DNA gel extraction

PCR-amplified and digested DNA fragments were separated by size using agarose gel electrophoresis [1 to 3% (w/v) agarose (depending on fragment size); 1 x TAE; 0.5 mg/ml EtBr; 1 x TAE electrophoresis buffer]. 1 x TAE electrophoresis buffer was used as running buffer.

2.2.1.4 RNA isolation

Frozen pieces of liver tissue were placed in a tube with ceramic beads. 1ml of Trizol was added and the tissue was homogenized in a tissue homogenizer (Precellys 24, PeqLab) at a speed of 6800rpm for 20 seconds. The homogenized samples were spun at 4 °C, 12000g for 10 minutes. The supernatant was taken and mixed with 200µl of Chloroform. The tubes were mixed vigorously for 15 seconds and incubated at room temperature for 2 minutes, then centrifuged for 15 minutes, at 4 °C, 12000g. The upper colourless phase containing the RNA

and DNA was transferred into a new tube and 600µl of 70% EtOH were added to yield a 1:1 dilution. The RNA and DNA were bound on columns from the RNEasy isolation Kit (Qiagen) and centrifuged 15 seconds, at 4 °C, 9000g. The column was washed once with 350µl of RW1 buffer followed by an on column DNA digestion with DNAse (Qiagen Dnase Kit) for 15 minutes at room temperature. After the DNAse digest the column was washed again with 350µl RW1, then twice with 500µl of RPE buffer. Between the washes centrifugation steps for 15 seconds, at 4°C, 9000g were carried out. After washing, the columns were placed in fresh collection tubes and spun at full speed for 2 minutes. The RNA was eluted twice in 30µl of RNase free water. The quality of isolated RNA was assessed by electrophoresis on denaturing agarose gels (1.2% (w/v) agarose; 1 x MOPS; 1% (v/v) formaldehyde; 1 x MOPSelectrophoresis buffer). To determine possible DNA contaminations a PCR for actin was performed with 1 µg of RNA.

2.2.1.5 cDNA synthesis

1µg of RNA was used to synthesize cDNA using the first strand RNA synthesis Kit (Invitrogen). The RNA was mixed with 1µl 10mM dNTP mix and 1µl 50ng/µl random hexamer mix. Water was added to a final volume of 10µl. The sample was incubated at 65°C for 5 minutes and placed on ice. The reaction mix containing 2µl of 10 RT buffer, 4µl 25mM MgCl2, 2µl 0,1 mM DTT, 1µl RNase out and 1µl SuperScript RT was added. The reverse transcription reaction was carried out starting with an incubation for 10 minutes at 25°C followed by 50 minutes at 50°C. The reaction was terminated at 85°C for 5 minutes. 1µl of RNase was added to digest the RNA at 37°C for 20 minutes. The cDNA was diluted in 180µl of water and successful cDNA synthesis was verified with a PCR for actin using 2µl of each cDNA sample.

2.2.1.6 Quantitative PCR

qPCR reactions were carried out in a 384 well plate. 2µl of cDNA, 3,4 µl of water, 0,6 µl of primer mix (Applied Biosystems) and 6 µl of Taqman master mix (Applied Biosystems) were used per reaction. Samples were run in duplicate with a non template control for each primer set. Dilutions, namely 1:4, 1:16, 1:64, with a positive cDNA were run as a control.

2.2.2 Cell biology

2.2.2.1 Isolation of primary hepatocytes

Prior to the isolation, the pump was rinsed with 70% Ethanol followed by a short rinse with deionized water. Solution I (EBSS without Ca^{2+} and Mg^{2+}, 0,5ml 0,5M EGTA), and solution II (EBSS with Ca^{2+} and Mg^{2+}, 5ml of 1M HEPES), were prepared, kept at 4°C and warmed up to 40°C in a water bath prior to use. 15mg of Collagenase and 2mg Trypsin inhibitor (Sigma) were placed in a 50 ml falcon tube. A cell culture centrifuge was cooled down to 4°C before starting the procedure.

The mice were anesthetized by intraperitoneal injection of Ketamin/Rompum (~12µl/g bw). Once the mice were unconscious and resistant to a toe pinge test, the peritoneal cavity was opened and the organs pushed aside in order to expose the *vena cava*. The boryle was inserted through the *vena cava* and the portal vein was cut prior to perfusion. The mice were perfused via the *vena cava* with solution I at a speed of 6 for 5 minutes. The tube containing the collagenase and trypsin inhibitor was filled with 50 ml of solution II. Subsequently, perfusion with 50 ml of collagenase (Worthington/Cell systems) solution (EBSS with Ca^{2+} and Mg^{2+}, 10 mM Hepes, 3810 U collagenase and 2 mg Trypsin inhibitor) was performed. After perfusing with 50ml collagenase solution at a speed of 4-5, the liver was taken out carefully and the gallbladder was excised. A single cell suspension from the perfused liver was generated by carefully breaaking the capsule of the liver and filtering the suspension through a 70 µm nylon mesh. Hepatocytes were washed 3 times in high glucose DMEM supplemented with 5% FCS. 3 x 10^6 cells were seeded on collagen coated 10 cm dishes. 4h after isolation, the medium was changed.

2.2.2.2 Hepatocyte culture

Primary hepatocyteswere cultured in DMEM GlutaMAXTM I (Gibco) supplemented with 5% (v/v) FCS (Gibco), 100 U/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), and 1 mM sodium pyruvate(Gibo) at 37°C in an atmosphere of 5% CO2 and 95% humidity. The medium was changed every day. Hepatocytes were cultures for a maximum of 2 days

2.2.2.3 Insulin Signaling *in vitro*

Primary hepatocytes were cultured over night. Cells were starved in serum free medium for 4

hours. 200mM of insulin (Sigma) were added to the cells for 2, 5 or 10 minutes. The cells were then washed with 10ml of ice cold PBS, scraped off the plate with 1ml of ice cold PBS, washed once with PBS and frozen in liquid nitrogen.

2.2.3 Biochemistry

2.2.3.1 Lysis of tissue samples with NP-40 Buffer

Tissue samples were homogenized in a tissue homogenizer (Precellys 24, PeqLab) using 600µl of NP-40 Buffer. Soft tissues including liver and fat were homogenized at 6800rpm for 20 seconds while muscle tissue was homogenized at 6800rpm for 2x20 seconds. During this process the samples were kept at 4°C. The cell debris was spun down at 13000rpm at 4°C for 10 minutes. A Bradford assay was used to determine the protein concentration of the samples.

2.2.3.2 Lysis of hepatocytes with high salt RIPA buffer

Cell pellet (from 10cm dish) was dissolved in 350µl of RIPA buffer, vortexed and incubated on ice for 30 minutes. The cell debris was collected by spinning at maximum speed for 20 minutes at 4°C.. Supernatant was taken off. The protein lysate was sonicated for 7 minutes 4°C in a water bath sonicator. A Bradford assay was used to determine the protein concentration of the samples.

2.2.3.3 Bradford assay

For the measurement of protein concentration, tissue samples were diluted 1:10 in lysis buffer. Standard BSA concentrations of 5, 2,5, 1,25 and 0,675 µg/µl were measured to generate a standard curve. 1 µl of each sample was mixed with 1 ml of Bradford solution, incubated for 5 minutes and measured with a spectrometer. Protein concentrations were calculated with the standard curve and samples with equal concentration were generated and boiled with loading dye at 96°C for 3 minutes.

2.2.3.4 Immunoblot analysis

Protein samples were separated on a 10% SDS-polyacrylamide gel by electrophoresis (PAGE) with a current of 100V. After separation, the protein was transferred to a nitrocellulose membrane that had been previously activated with methanol, at a maximum current of 20V (80mA per gel) for 2 hours, using a semi.dry blotting system. For proteins >100 kDa a wet blotting system was used transferring the protein at a current of 80V for 2,5 hours. The membranes were probed with antibodies specific for: Tubulin α (Sigma), p38α, IKK2, c-jun, phospho-c-jun, JNK, phospho -JNK, cleaved caspase 3, total caspase 3, phospho -MKK7, MKK7, phospho -MKK4, MKK4, MKK3, phospho -MKK3/6, p38δ, IκBα, phospho -Akt, Akt, phospho -ERK1/2, ERK1/2 (Cell Signaling), c-FLIP (Alexis), p38β, phospho-IκBα, MKP1, MKP2, MKP3 (Santa Cruz) pp5 (BD), pp1me (ABGENT), pp1a (Biozol), pp2a (Upstate). HRP-conjugated anti-rabbit, anti-mouse and anti-rat secondary antibodies were used (Amersham).

2.2.3.5 Stripping of PVDF membranes

The membranes were washed with deoinized water for 5 minutes shaking at room temperature, incubated for max. 5 minutes in 0,2M NaOH and washed with deoinized water twice for minutes.

2.2.3.6 Electromobility shift assay (EMSA).

In order to investigate Protein-DNA interactions, nuclear proteins were incubated with a radioactively labeled oligonucleotide and subsequently separated by electrophoresis. The sequence of the oligonicleotide was chosen to have a specific binding sequence for the protein. In this case a 32P-labelled oligonucleotide representing an NF-κB consensus site (5'-CGG GCT GGG GAT TCC CCA TCT CGG TAC-3') was used as a probe. In order to be able to identify the complexes on the gel, antibodies which are able to bind the protein-DNA complex were used. The Protein-DNA complexes were then identified by their altered migration on the gel (supershift). For each sample 2-5 µg of nuclear protein was used. Protein samples were incubated in a reaction mix with a total reaction volume of 20µl containing 5µl 5x binding buffer, 1µl Poly(dI-dC), 1µl BSA 10 µg/µl indH2O, 2 µl 10x proteinase-inhibitor cocktail and 50.000cpm [32P] labeled Oligonucleotide for 30 minutes on ice. For supershift analysis antibodies were added after the incubation for 30 minutes (p50, p65, c-Rel and RelB from Santa Cruz Biotechnology). Then, 2µl loading buffer (20% Ficoll suspension) were added and the probes were loaded on the 5% acrylamide gel. Samples

were run at 4°C. After 20 minutes the current was raised from 200V to 300V. The separation time was 3 hours in total. The gel was fixed for 15 minutes in 20% Methanol/10% acetic acid and subsequently dried under vacuum. The dry gel was exposed to a film at -80°C for several days.

2.2.3.7 Histology

Cryo sections

For cryo sections tissues were embedded in tissue freezing medium (Jung Tissue Freezing medium, Leica Microsystems) and frozen on dry ice. Later, the frozen blocks were transferred to -80°C. Prior to sectioning the frozen blocks were placed at - 20°C for several hours. Standard thickness for paraffin sections was 7µm. For Oil red O liver stainings 12µm sections were cut.

Paraffin sections

Tissues were fixed in 4% paraformaldehyde over night at 4°C. After fixation the samples were placed in PBS at 4°C until embedding in Paraffin.

H&E Staining

Sections were deparaffinized in Xylol for 20 minutes. Then the samples were hydrated in 100% Ethanol for 2 minutes, followed by 2 minutes of 90% Ethanol and 2 minutes of 75% Ethanol. The sections were washed in PBS briefly and 1 minute in tap water. Next, sections were incubated in Haematoxylin for 6 minutes (liver) followed by a short wash in warm water and a short wash in deionized water. The samples were incubated in Eosin solution for 1 minute, then washed 6 to 7 times in tap water. Next, sections were dehydrated in 75% Ethanol for 1 minute, followed by 1 minute of 90% Ethanol and 1 minute of 100% Ethanol. The sections were then incubated in Xylol for 1-5 minutes and mounted with Eukitt.

Oil red O staning

Cryo sections were washed in tap water for 5 minutes. After washing the sections were stained with Oil red O solution for 15 minutes then quickly rinsed with tab water. The sections were counterstained for 6 minutes with Haematoxylin solution. The stained sections were washed with water for 15 minutes then mounted with Glyceringelatine.

2.2.3.8 TUNEL assay

The TUNEL test was performed using the "*In situ* cell death detection Kit" (Roche Diagnostics) Sections were fixed in 4% PFA dissolved in PBS for 30 minutes at room temperature. After fixation the sections were washed twice in PBS (once short, than 5 minutes). Next, the sections were incubated with Proteinase K (from Kit) diluted 1:1000 in Tris/EDTA buffer (100mM Tris pH 8/HCL 80mM EDTA) using a moist chamber for 30 min at room temperature. After the Proteiniase K digest, the sections were washed 2 x in PBS for 5 minutes and then permeabilized with Triton (0,1%Triton in 0,1% Na-Citrat) for 15 minutes. The sections were then washed again 2 x in PBS for 5 minutes. Excess liquid was removed and slides were equilibrated with 100 ul of Equilibration Buffer for 10 minutes. While the cells were equilibrating, the reaction mix was prepared on ice: Equilibration Buffer 45 ul, Nucleotide Mix 5 ul, TdT Enzym1µl (per reaction). The mix was added on the slides which were covered with Plastic Coverslips (from Kit) for 60 minutes at room temperature in a chamber with aluminum foil. The slides were then incubated in 2x SSC for 15 minutes to terminate the reactions. After terminating the reaction, the slides were washed 3x in PBS for 5 minutes, then mounted with Vectashild and DAPI.

2.2.3.9 Lipid Analysis (Thin layer Chromatography)

The mouse liver content of cholesterol, cholesteryl esters and triacylglycerols was analyzed by Thin Layer Chromatography. Samples of mouse liver tissue (50 to 150 mg) were homogenized with the Homogenisator Precellys 24 (Peqlab) in 1 ml of water at 6.500 rpm for 30 seconds. The protein content of the homogenate was routinely determined using bicinchoninic acid. After addition of 4 ml of methanol and 2 ml of chloroform, lipids were extracted for 24 h at 37°C. The liquid phase was separated by filtration, and the insoluble tissue residues were further extracted for 24 h at 37°C in 6 ml of methanol/chloroform 1:1 (v/v) and finally 6 ml of methanol/chloroform 1:2 (v/v). The extracts were combined, and the solvent was evaporated under a stream of nitrogen. The residues were purified using a modification of the Bligh-Dyer procedure as previously described (Signorelli and Hannun 2002). Lipids were applied to 20 × 10 cm high performance thin layer chromatography (HPTLC) Silica Gel 60 plates (Merck), which were pre-washed twice with chloroform/methanol 1:1 (v/v) and air-dried for 30 min. For quantification of triacylglycerols and cholesteryl esters, each lane of the TLC plate was loaded with the equivalent of 50 µg and 1 mg wet weight of liver tissue, respectively. The TLC solvent system used was hexane/toluene 1:1 (v/v), followed by hexane/diethyl ether/glacial acetic acid 80:20:1 (v/v). For quantitative analytical TLC determination, increasing amounts of standard lipids (Sigma-

Aldrich) were applied to the TLC plates in addition to the lipid samples. For detection of lipid bands, the TLC plates were dipped into a phosphoric acid/copper sulfate reagent (15.6 g of CuSO4(H2O)5 and 9.4 ml of H3PO4 (85%, w/v) in 100 ml of water) and charred at 180°C for 10 min (Yao and Rastetter 1985). Lipid bands were then quantified by densitometry using the TLC-Scanner 3 (CAMAG), at a wavelength of 595 nm.

2.2.4 Mouse analysis

2.2.4.1 Animal care

Care of all animals was within institutional animal care committee guidelines. All animal procedures were conducted in compliance with protocols and approved by local government authorities (Bezirksregierung Köln, Cologne, Germany). Animals were either fed normal chow (Teklad Global Rodent 2018; Harlan) containing 53.5% carbohydrates, 18.5% protein, and 5.5% fat (12% of calories from fat) or a HFD (C1057; Altromin) containing 32.7% carbohydrates, 20% protein, and 35.5% fat (55.2% of calories from fat). All mice were kept in individual ventilated cages (IVC) in the animal facility of the Institute for Genetics in Cologne. All experiments were performed according to EU, national and institutional guidelines. For the metabolic project bodyweight was measured once a week.

2.2.4.2 Generation of conditional knockout mice

p38α^{Fl} (Heinrichsdorff et al., 2008), NEMO^{Fl} mice (Schmidt-Supprian et al., 2000) and IKK2^{Fl} mice (Pasparakis et al., 2002) were crossed to Alfp-Cre transgenic mice (Kellendonk et al., 2000) to generate liver parenchymal cell-specific knockout of the respective gene (p38$\alpha^{LPC\text{-}KO}$, NEMO$^{LPC\text{-}KO}$ and IKK2$^{LPC\text{-}KO}$ mice). p38α/IKK2 double mutant mice were generated by crossing p38$\alpha^{LPC\text{-}KO}$ mice to IKK2$^{Fl/Fl}$ mice (p38α/IKK2$^{LPC\text{-}KO}$).

2.2.4.3 Genotyping of mutant mice

PCR reaction volume: 30 µl

	1 reaction
Buffer 10x	3 µl

dNTPs 2mM	3 µl
Primer mix 10x (33 µM each)	3 µl
MgCl2 25mM	1,8 µl (f.c.1,5mM)
DNA	2 µl
Taq 5 U/µl	0,2 µl
dd-Water	17 µl

Program Alfp cre/IKK2:

94°C	3 min	
94°C	30 sec	
60°C	30 sec	35x
72°C	30 min	

Program p38α :

94°C	3 min	
94°C	30 sec	
62°C	30 sec	35x
72°C	30 sec	
72°C	2 min	

2.2.4.4 Liver injury models

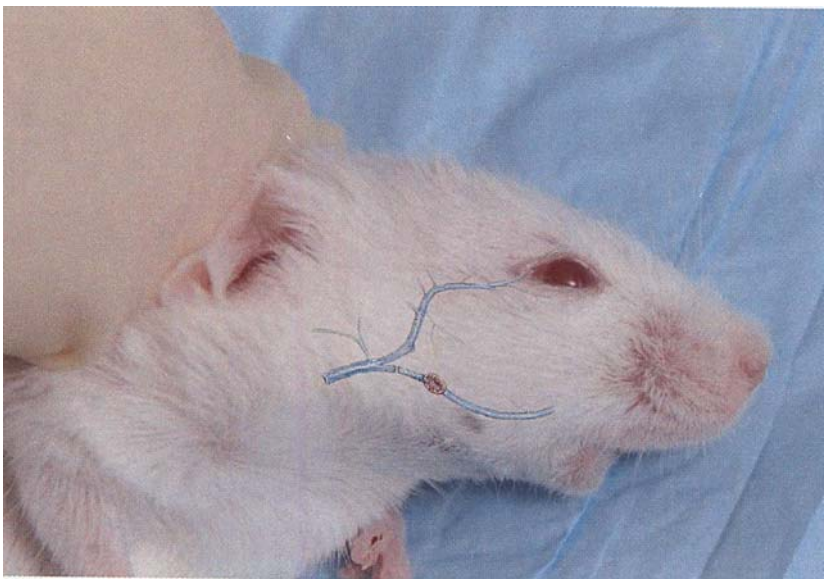
Experiments were performed on male mice between 8 and 10 weeks of age. LPS (Sigma) was administered i.p. at 25µg/10g of body weight. Mice were bled before and 4 hours after the injection by submandibular bleeding. 10 hours after the injection the mice were sacrificed and blood was taken from the heart.

2.2.4.5 Antioxidant treatment

For antioxidant treatment mice were fed a diet containing 0,7% butylated hydroxyanisole (BHA) (Sigma) for 3 days before LPS injection.

2.2.4.6 Submandibular bleeding

The superficial temporal vein runs diagonally across the face from above the orbit toward the base of the ear.



The animals were manually restrained with the lateral surface of the hand facing the person holding the mouse. The lancet (Goldenrod Animal Lancet) was held in the other hand. A stab incision into the cheek was made, approximately halfway between the ear and the mandible. The blood was collected into a tube. After bleeding the mouse was released from the restrain and a compression was applied to the cheek for 5 seconds. After the procedure the mice were monitored for 5 minutes to ensure that the bleeding had stopped. For mice <20 grams 4mm lancets were used, for mice between 20-40 grams 5mm lancets and for mice >40 grams 6mm lancets were used.

2.2.4.7 Serum analysis

Mice were bled from the tail vein or by submandibular bleeding. The blood was stored at 4 °C for 10 minutes then centrifuged for 5 minutes at 6000g at 4°C. The serum was taken off and analyzed with the Cobas 1001 Clinical analyzer (Roche) (GOT, GPT, Chlesterol, Triazylglycerides) or by ELISA (insulin).

2.2.4.8 Glucose tolerance test

Glucose tolerance tests were performed on animals that had been fasted for 16 hours. Animals were injected with 2g/kg bodyweight of glucose into the peritoneal cavity. Blood glucose concentrations were determined before, 15, 30 60 and 120 minutes after the injection of glucose. Blood was taken from a small tail biopsy and glucose content was measured using an automated glucose monitor (Glucoman) for the indicated time points.

2.2.4.9 Insulin tolerance test

0,75 U/kg bodyweight of human regular insulin (Insuman Rapid, Sanofi Aventis) was injected into the peritoneal cavity. Blood glucose concentrations were determined before, 15, 30 60 and 120 minutes after the injection of glucose. Blood was taken from a small tail biopsy and glucose content was measured using an automated glucose monitor (Glucoman) for the indicated time points.

2.2.4.10 Insulin signaling, *in vivo*

Mice were anesthetized by intraperitoneal injection of Ketamin/Rompum (~12µl/g bw) 5 mU. The peritoneal cavitiy was opened to expose the *vena cava*. Human regular insulin (Insuman Rapid, Sanofi Aventis) was injected into the inferior *vena cava*. Liver, muscle and adipose tissue were collected 2, 5 and 7 minutes after injection and processed as previously described to determine insulin mediated signaling by immunoblot analysis.

2.2.4.11 Measurement of serum insulin concentrations.

Plasma insulin concentrations were examined by ELISA (Chrystal Chem, BD Bioscience PharMingen). Each well of the microplate was washed twice with wash buffer. 50µl guinea pig anti-insulin serum were dispensed per well. Then, 45µl of sample diluent and 5µl of sample (or 5µl working insulin standard) were given in the wells. The microplate was incubated overnight at 4 °C. After incubation, the wells were washed three times with wash buffer. 100 µl of anti-guinea pig antibody enzyme conjugate were given into every well, followed by incubation for 3 hours at room temperature. The wells were then washed 5 times with wash buffer solution. 100µl of enzyme substrate solution were dispensed per well. The microplate was incubated for 30 minutes at room temperature while avoiding exposure to light. The reaction was stopped by adding 50µl of enzyme reaction stop solution per well and

the plate was measured immediately at A_{492}. A_{630} values were subtracted. Insulin concentration was calculated by the standard curve.

2.2.4. Analysis of body composition by NMR

Lean mass and body fat content of live animals was determined using the NMR Analyser Minispec (Bruker Optik).

2.2.5 Statistics.

Results are expressed as the mean +/- standard error of the mean (SEM). Statistical significance between experimental groups was assessed using an unpaired two-sample Student t-Test.

3 Results

3.1 Generation of mice with a liver parenchymal cell specific deletion of p38α

In order to delete p38α specifically in hepatocytes $p38\alpha^{FL}$ mice were crossed with mice carrying the Alfp cre transgene (Reichardt, Kellendonk et al. 1999). The $p38\alpha^{FL}$ mice carry loxP sites flanking exons 2 and 3, which include the ATP-binding site of the kinase domain (Fig. 1 A). At this point it should be mentioned that “FL” is the term for “floxed” which is used in this thesis and describes mice carrying loxP sites in both alleles. Cre is expressed under the control of both the mouse albumin regulatory elements and the α-fetoprotein enhancer and leads to cre expression exclusively in hepatocytes and bile duct epithelial cells. By crossing $p38\alpha^{FL}$ mice with Alfp cre positive mice $p38\alpha^{LPC\text{-}KO}$ mice were obtained that had a deletion of p38α specifically in hepatocytes and bile duct epithelial cells.

$p38\alpha^{LPC\text{-}KO}$ mice were born at the expected Mendelian ratio, were viable and fertile, and did not show signs of hepatic alterations. The generation of $p38\alpha^{LPC\text{-}KO}$ mice results in efficient deletion of p38α in the liver of these mice which was shown by immunoblot analysis (Fig. 1 B). However, some expression of p38α was retained owing to the presence of non-parenchymal cells such as Kupffer and endothelial cells that are not targeted by the Alfp-Cre transgene. In order to check if there was compensatory elevation of p38ß and p38δ, immunoblot analysis of liver extracts from $p38\alpha^{LPC\text{-}KO}$ and wild type mice was performed. As seen in Fig. 1 C, expression levels of p38 δ did not change due to the p38α knockout, while p38ß was not detected.

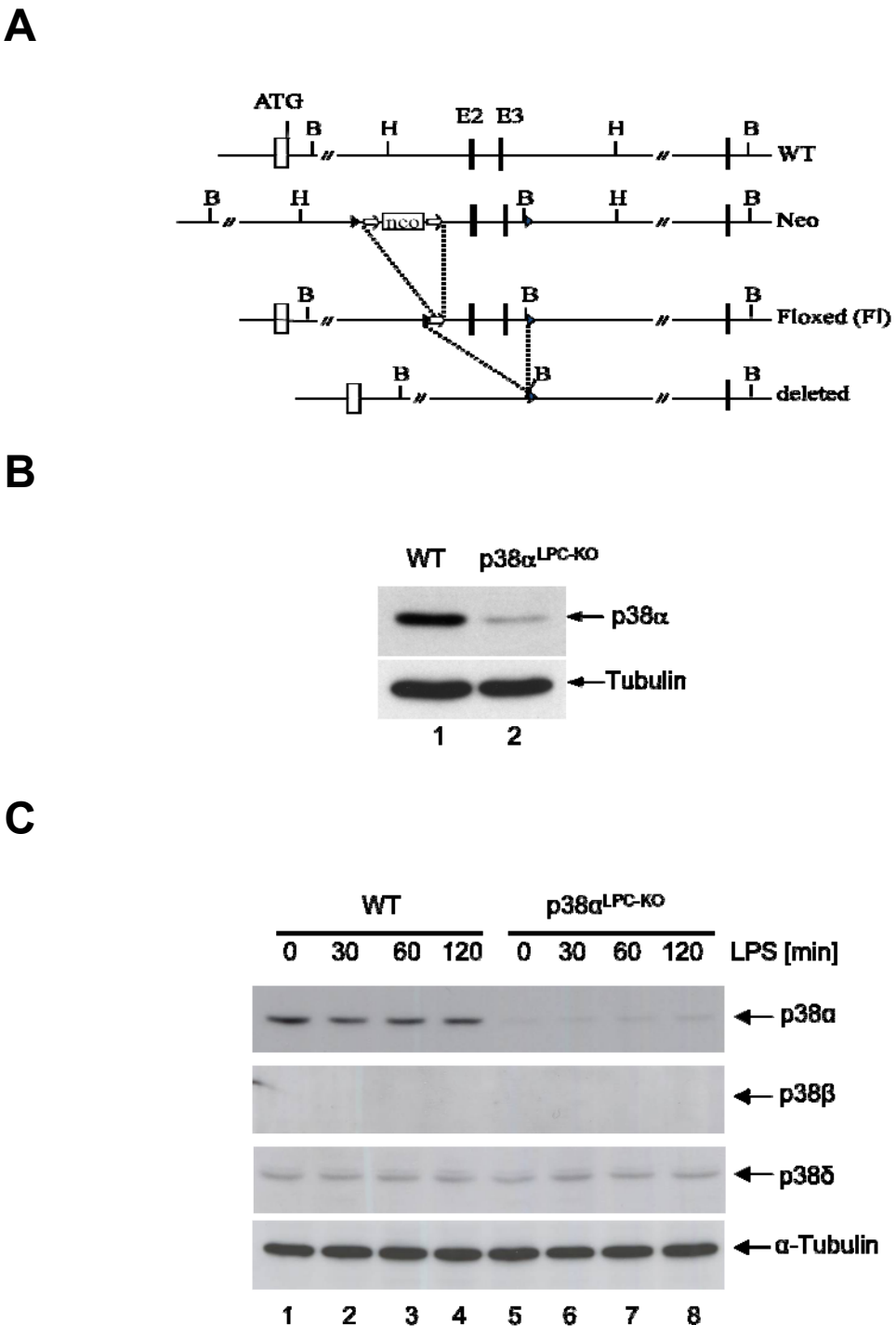


Figure 1: Liver parenchymal cell specific deletion of p38α. (A) Schematic description of the targeting strategy for the generation of mice with loxP-flanked p38α alleles. Filled boxes indicate the loxP-flanked exons 2 and 3 (E2, E3), which include the ATP-binding site of the kinase domain. B, BamHI; H, HindIII. Black arrowheads indicate loxP sites; white arrows indicate FLP recombinase target (Frt) sites. (B) Immunoblot analysis of p38α expression in liver extracts from wild-type (WT) and p38α^{LPC-KO} mice. (C) Immunolot analysis of p38ß and p38δ in liver extracts from wild-type (WT) and p38α^{LPC-KO} mice.

3.2 The role of liver p38α in a model of LPS/TNF induced liver toxicity

3.2.1 p38α^{LPC-KO} mice are not sensitive to LPS-induced liver failure

In order to examine the function of liver p38α in cytokine induced signaling, p38α^{LPC-KO} and littermate control mice were injected with 25µg/10g LPS into the peritoneal cavity. For all experiments described here, littermates carrying loxP-flanked p38α alleles but lacking expression of Cre recombinase were used and were termed wild-type or control animals.
Liver damage was assessed by measuring serum aminotranferases which are quickly released into the circulation upon hepatocyte death (Fig. 2 A). Moreover hepatocyte apoptosis was measured by TdT-mediated dUTP nick end labelling (TUNEL) of liver sections (Fig 2 B) and by immunoblot analysis of caspase 3 activation in liver extracts (Fig. 2 C). Figures 2 A-C clearly show that the deletion of p38α does not lead to increased hepatocyte apoptosis compared to control animals after LPS injection. In contrast to that, as shown previously (Luedde, Beraza et al. 2007), the deletion of NEMO in liver parenchymal cells results in much higher sensitivity to LPS induced hepatocyte death (Fig. 2 B and C)

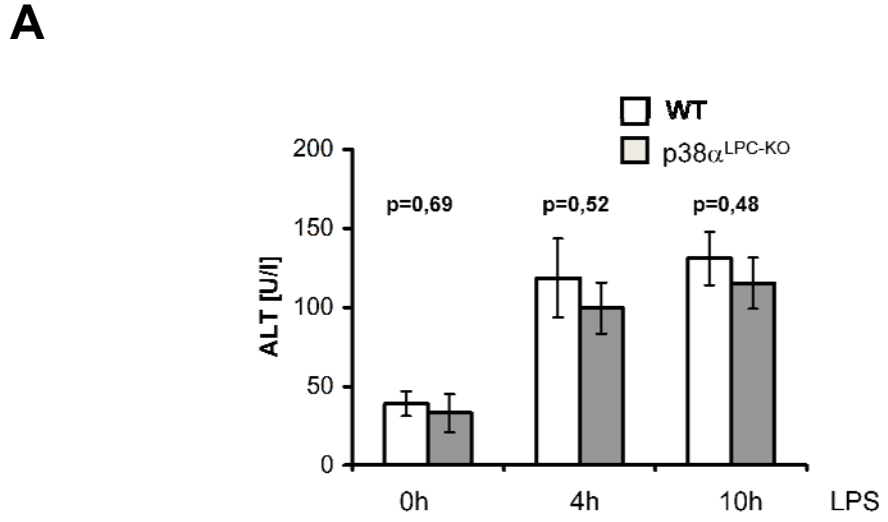


Figure 2: p38α^{LPC-KO} mice are not sensitive to LPS-induced liver failure. Assessment of liver damage in (A) p38α^{LPC-KO} and control mice after LPS injection. Levels of free circulating alanine aminotransferases (ALTs) were measured in the serum of p38α^{LPC-KO} and control mice at the indicated time points after LPS injection. Error bars denote s.e.m. (n>4).

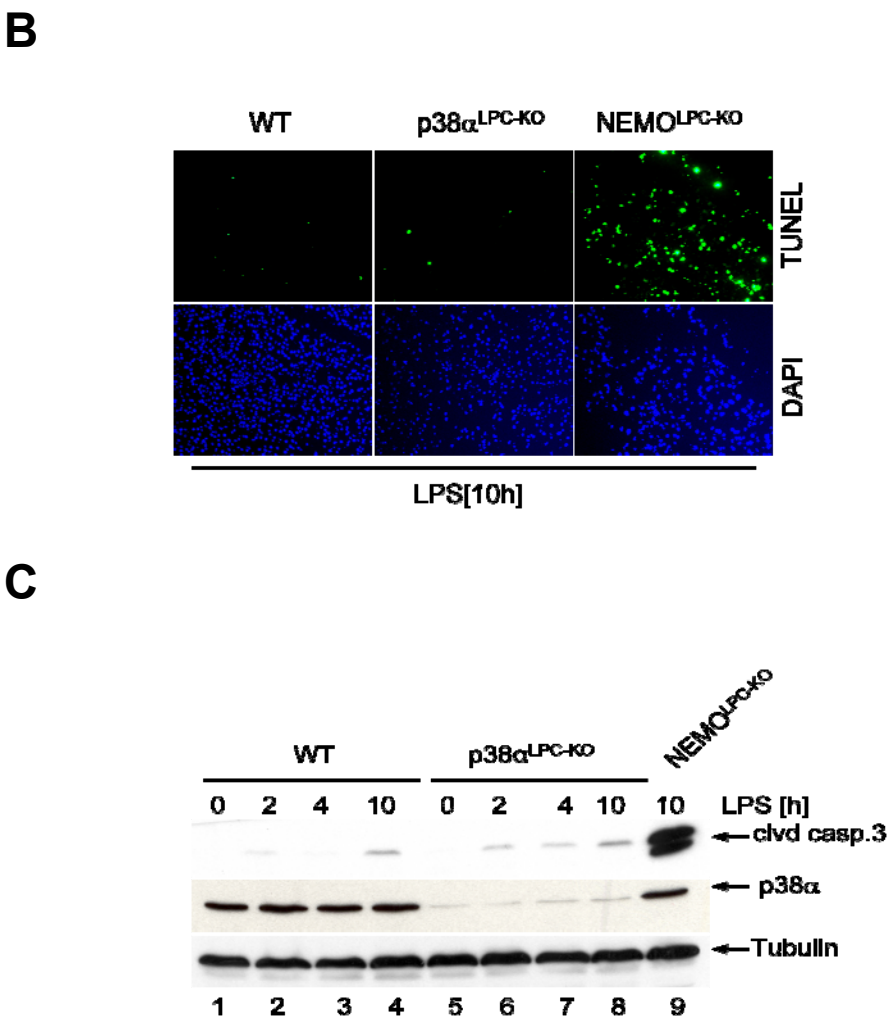


Figure 2: p38α^{LPC-KO} mice are not sensitive to LPS-induced liver failure. (B) Detection of apoptotic cells by TUNEL assay in livers from wild-type, p38α^{LPC-KO} and NEMO^{LPC-KO} (used as a positive control) mice 10 h, after LPS injection. (C) Immunoblot analysis of caspase 3 activation using an antibody that specifically detects the cleaved form in protein extracts generated from WT and p38α^{LPC-KO} mice. NEMO^{LPC-KO} mice that are characterized by complete inhibition of canonical NF-κB signaling in the liver, are used as positive control (Fig 2 B, C).

3.2.2 Increased activation of JNK in p38α-deficient livers

Sustained activation of JNK has been shown to be essential for TNF -induced killing of NF-kB-deficient liver cells (Kamata et al, 2005; Chang et al, 2006). Therefore, the status of JNK activation in p38α-deficient and control livers was investigated after LPS injection. Surprisingly, JNK activation was strongly increased in livers from p38α^{LPC-KO} mice compared with control mice after LPS administration (Fig. 3 A). In contrast, NEMO-deficient livers that are highly apoptotic, did not show a hyperactivation of JNK compared to control livers (Fig. 3 B). Consistent with JNK activation, c-Jun phosphorylation was also induced in livers from p38α^{LPC-KO} mice after LPS injection (Fig. 2 C). Thus, although pJNK levels are massively

increased in the livers of p38α^{LPC-KO} mice after LPS injection, these livers are not sensitized to LPS induced toxicity. Thus, sustained JNK activation in response to TNF signaling does not seem to be sufficient to cause hepatocyte death in p38α deficient livers.

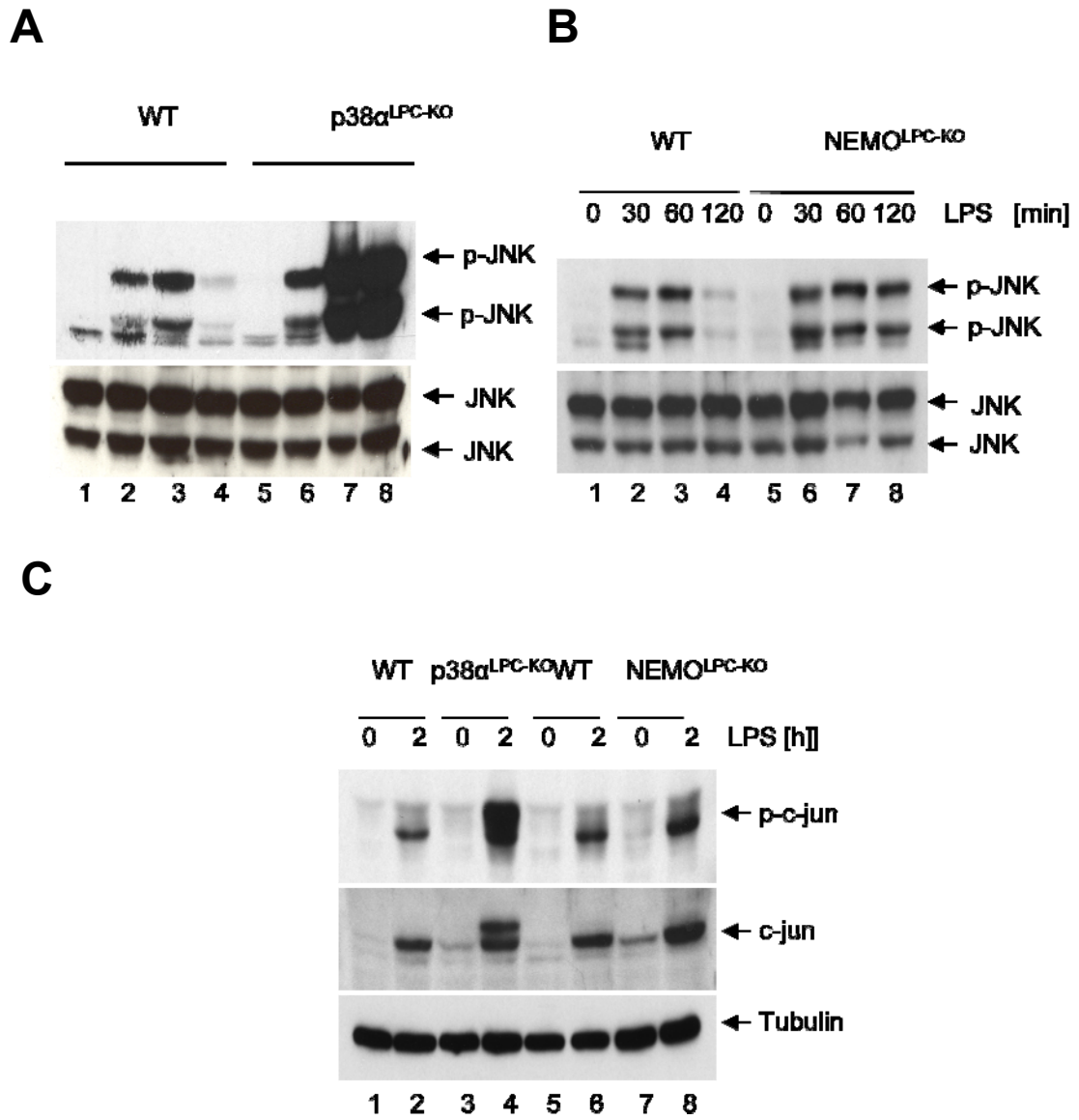


Figure 3: Increased activation of JNK in p38α-deficient livers. (A) Phosphorylation of JNK was assessed in liver extracts from wild-type and p38α^{LPC-KO} mice that were injected with 25µg/10g body weight LPS for the indicated times, by immunoblot analysis using phospho-JNK-specific antibodies (upper panel). JNK immunoblot acts as loading control. (B) Immunoblot analysis detecting the phosphorylation of JNK in NEMO^{LPC-KO} and control (WT) mice. (C) Immunoblot analysis with antibodies recognizing phosphorylated c-Jun (upper panel), total c-Jun (middle panel) and Tubulin (lower panel) as loading control.

3.2.3 LPS does not induce increased activation of the NF-κB, ERK and AKT survival pathways in p38α^{LPC-KO} livers

In order to assess if an increased activation of survival pathways contributes to the survival of hepatocytes in p38α deficient cells despite massive activation of JNK, the activation of NF-κB, extracellular signal-regulated kinase (ERK) and AKT was measured. Activation of NF-κB, shown by immunoblot analysis of IκBα phosphorylation and degradation was similar in the livers of LPS injected p38α^{LPC-KO} and control mice (Fig. 4 A). Moreover, neither the activation of ERK, nor the activation of AKT in response to LPS injection differed between p38α^{LPC-KO} mice and control mice (Fig. 4 A and B)

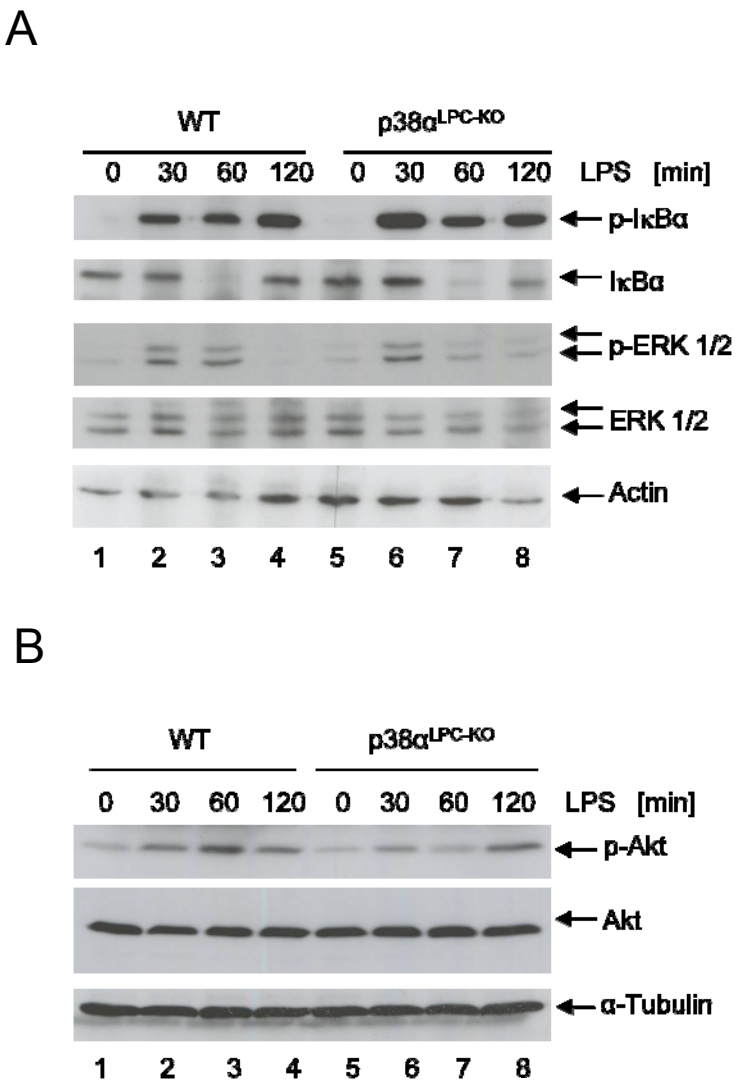


Figure 4: LPS does not induce increased activation of the NF-κB, ERK and AKT survival pathways in p38α^{LPC-KO} livers. (A) Immunoblot analysis of liver extracts from wild-type and p38α^{LPC-KO} mice after LPS injection at the indicated time points assessing NF-κB and ERK activation using antibodies against the phosphorylated form of pIκBα, IκBα, pERK 1/2 and ERK. Actin serves as a loading control. (B) Specific antibodies were used to detect pAKT to measure AKT activity after LPS injection. Tubulin and AKT serve as loading control.

3.2.4 Decreased c-Flip (L) levels in the livers of NEMO^{LPC-KO} mice, but not in p38α^{LPC-KO} mice

In order to find the reason why LPS induced TNF signaling is not able to kill p38α-deficient hepatocytes despite the massive activation of proapoptotic JNK, the effects of p38α ablation on downstream mediators of JNK function were examined. Previously, it was reported that JNK facilitates TNF-induced apoptosis by promoting the proteasome-mediated degradation of the long c-FLIP isoform c-FLIP (L), an inhibitor of caspase 8, the expression of which is regulated by NF-κB (Micheau, Lens et al. 2001; Chang, Kamata et al. 2006). Immunoblot analysis was performed on livers taken from p38α^{LPC-KO}, NEMO^{LPC-KO} and control mice after LPS injection. c-FLIP(L) levels were assessed, showing a slight down regulation of c-FLIP (L) protein 4 h after LPS injection in livers taken from p38α^{LPC-KO} mice compared to livers from control mice (Fig 5 A). However, 10 h after injection of LPS, the levels of c-FLIP(L) were similar in p38α^{LPC-KO} and control mice (Fig 2 A). By contrast, the levels of c-FLIP (L) were diminished in NEMO deficient livers 10 h after LPS injection (Fig. 5 A, lanes 7–9), which is consistent with the increased hepatocyte apoptosis detected at this point. These results show that even a massive activation of JNK is not sufficient to reduce intracellular levels of c-FLIP (L) below the threshold required to sensitize hepatocytes to TNF-induced death. On the other hand, even a moderate prolongation of JNK activity results in degradation of c-FLIP(L) and apoptosis of hepatocytes with defective activation of NF-κB.

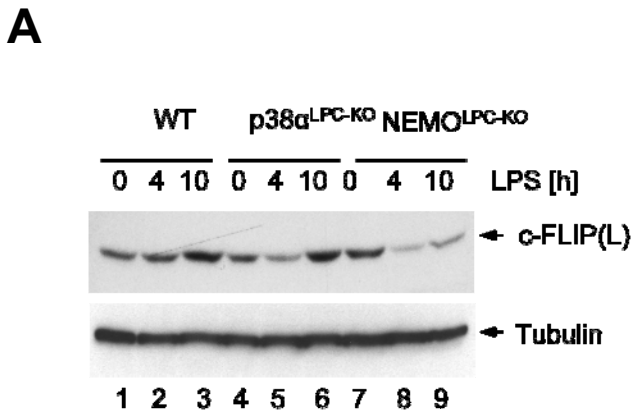


Figure 5: Decreased c-Flip levels in the livers of NEMO^{LPC-KO} mice, but not in p38α^{LPC-KO} mice. The levels of c-FLIP (L) in WT, p38α^{LPC-KO} and NEMO^{LPC-KO} mice were analyzed by immunoblotting at the indicated time points after LPS injection using specific antibodies against c-Flip (L). Tubulin acts as loading control.

3.2.5 Oxidative stress and MAP kinase phosphatases are not involved in the hyperactivation of JNK in livers of LPS injected p38α^{LPC-KO}mice

In NF-κB-deficient hepatocytes, accumulation of reactive oxygen species is one of the main contributing factors that leads to the up regulation of JNK caused by TNF, which can lead to the oxidation of JNK phosphatases (Kamata et al, 2005). To test the influence of oxidative stress, the antioxidant compound butylated hydroxyanisole (BHA) was administrated in the chow diet 48 hours before LPS injection.

Nevertheless, the BHA treatment did not lead to a substantial reduction of LPS-induced JNK activation in p38α^{LPC-KO} mice (Fig. 6 A), suggesting that the mechanism leading to increased JNK activation in p38α-deficient livers does not depend on oxidative stress. In addition, immunoblot analysis detecting a panel of phosphatases that have previously been reported to dephosphorylate JNK showed no differences in protein levels between control and p38α-deficient livers (Fig. 6 B).

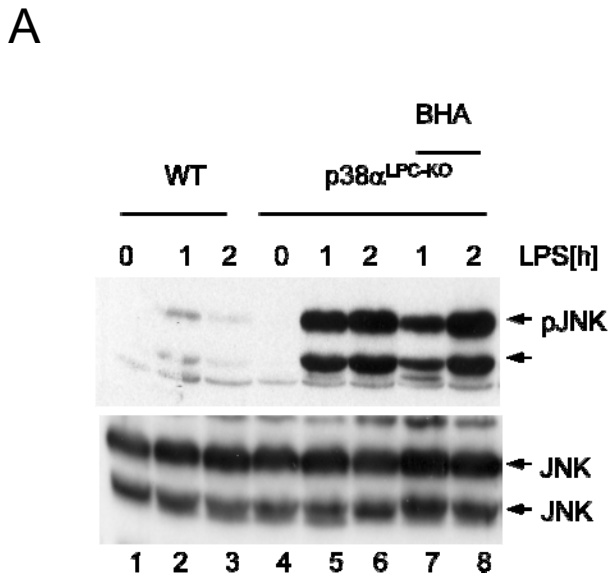
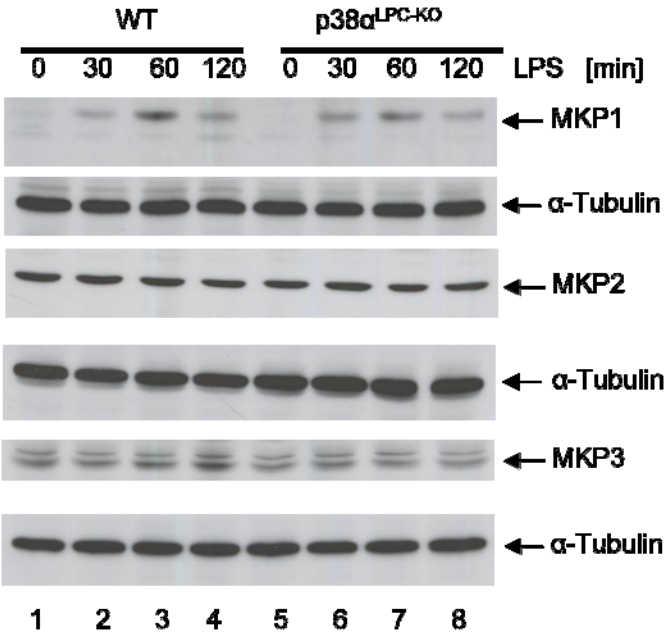


Figure 6: Oxidative stress and MAP kinase phosphatases are not involved in the hyperactivation of JNK in livers of LPS injected p38α^{LPC-KO}mice (A) Control (WT; lanes 1–3), p38α^{LPC-KO} (lanes 4–6) and p38α^{LPC-KO} mice that had been pretreated with the antioxidant compound BHA (lanes 7 and 8) were injected with LPS and sacrificed at the indicated time points. Immunoblot analysis was carried out on extracts of liver protein using antibodies against phosphorylated JNK or total JNK proteins.

B



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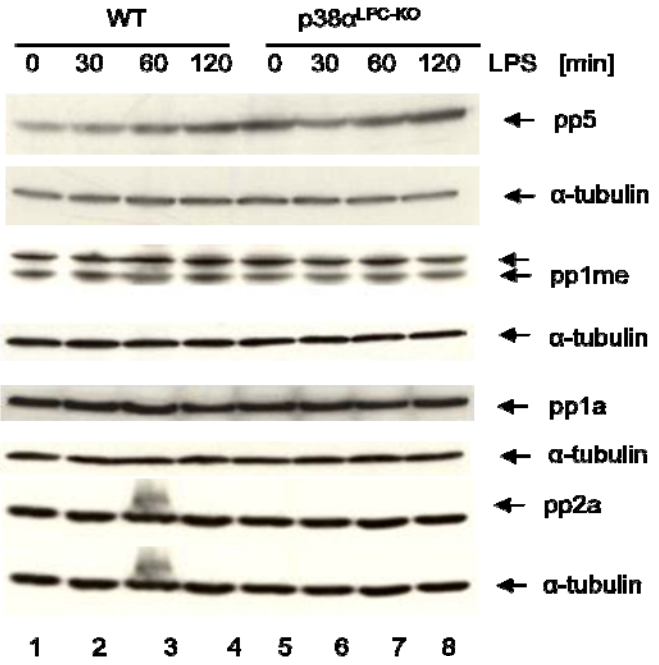

Figure 6: (C) Analysis of phosphatase expression in livers from control and p38α^{LPC-KO} mice. (B) Hepatocyte expression of MAP kinase specific phosphatases MKP1, MKP2 and MKP3 at steady state and after LPS injection at the indicated time points was assessed by immunoblot analysis using specific antibodies. (C) Hepatic expression of phosphatases involved in stress responses. Expression levels of pp5, pp1me pp1a and pp2a were

monitored using immunoblot analysis at steady state levels and after injection of LPS at the indicated time points. Tubulin serves as loading control.

3.2.6 LPS induced hyperactivation of MKK4, MKK3/6 and MLK3 in livers of p38α^{LPC-KO} mice

The phosphorylation of JNK is controlled by two upstream kinases, MKK7 and MKK4. MKK7 was reported to specifically act on JNK and to be activated by TNF and environmental stress.

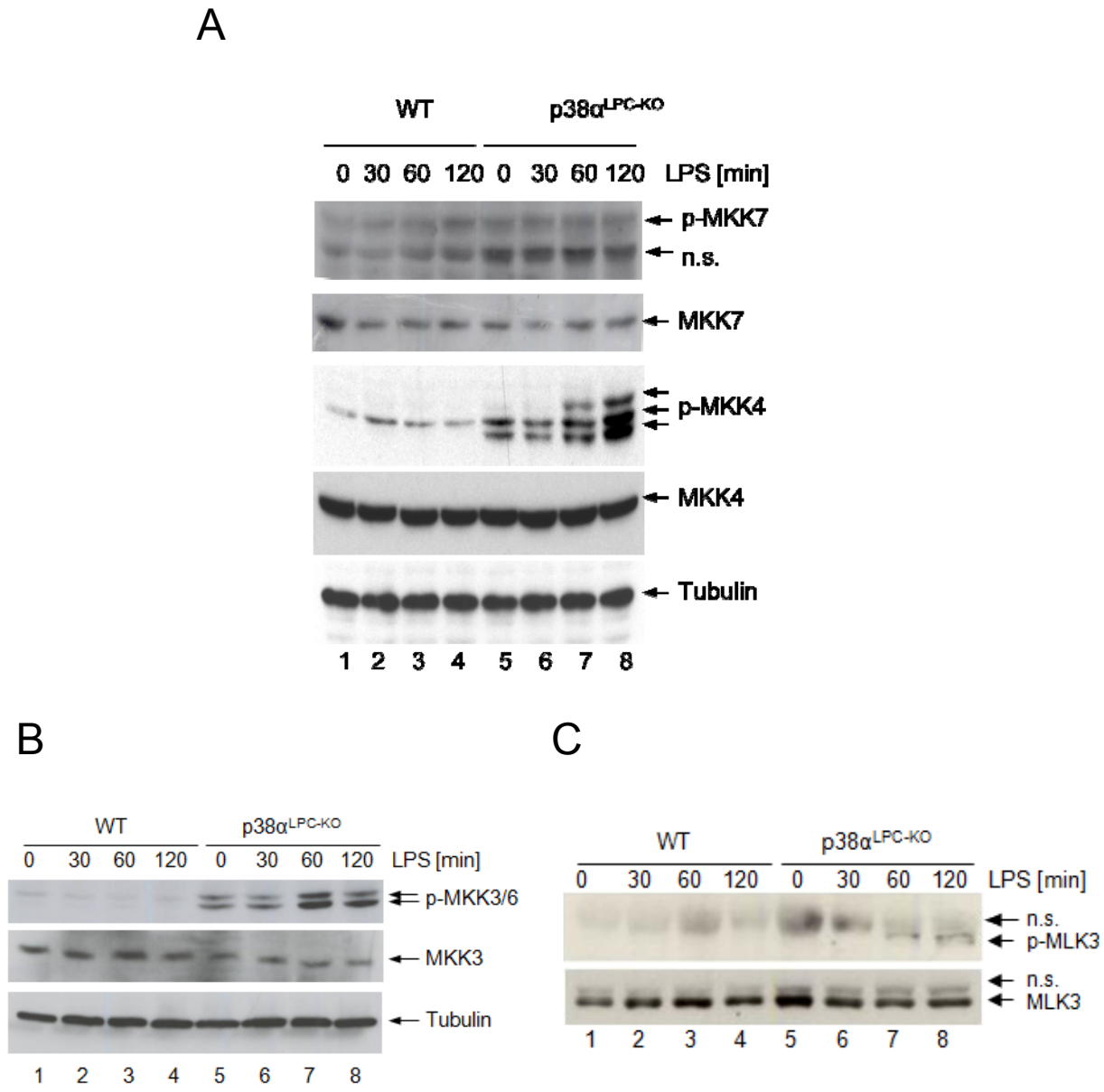

Figure 7: LPS induced hyperactivation of MKK4, MKK3/6 and MLK3 in livers of p38α^{LPC-KO} mice (A) Immunoblot analysis of the JNK-activating kinases MKK4 and MKK7 in the liver extracts from control and p38α^{LPC-KO} mice after LPS injection at the indicated time points. Tubulin acts as loading control. (B) Immunoblot analysis using an antibody detecting the phosphorylated forms of MKK3 and MKK6 (upper panel). (C) Immunoblot analysis using an antibody detecting the phosphorylated form of MLK3 (upper panel). Total MLK3 acts as loading control.

Assessment of MKK4 and MKK7 activation using immunoblot analysis with antibodies specifically recognizing their phosphorylated forms showed increased phosphorylation of MKK4, but not MKK7, in the liver of p38α^{LPC-KO} mice compared with wild-type mice after LPS injection (Fig. 7 A). Further analysis showed increased phosphorylation of the p38α-activating kinases MKK3/6 in livers from LPS-injected p38α^{LPC-KO} mice (Fig. 7 B). Interestingly, MKK4 was reported to phosphorylate both JNK and p38 MAPK, and is normally not activated by TNF (Derijard, Raingeaud et al. 1995; Minden, Lin et al. 1995; Moriguchi, Toyoshima et al. 1997; Tournier, Whitmarsh et al. 1999). Moreover the MAP3K MLK3 was found to be upregulated in livers from LPS-injected p38α^{LPC-KO} mice (Fig. 7 C).

3.2.7 p38α and IKK2 collaborate to prevent liver failure

Despite massive JNK activation p38α-deficient livers are not sensitive to LPS/TNF challenge. This is most likely due to the NF-κB dependent transcriptional induction of anti-apoptotic genes such as c-FLIP (L) which seems to be sufficient to prevent TNF-induced apoptosis of hepatocytes lacking p38α. To test this hypothesis, p38α^{LPC-KO} mice were additionally crossed with IKK2^{FL} mice which alone are not sensitive to LPS/TNF-induced liver failure (Luedde, Assmus et al. 2005) (Luedde, Beraza et al. 2007). The efficient deletion of p38α and IKK2 in liver parenchymal cells was shown in Figure 8 A. p38α/IKK2^{LPC-KO} mice were challenged with LPS and showed increased liver damage compared to single-mutant mice, shown by the analysis of serum alanine aminotransferase levels (Fig. 8 B). Measurement of apoptosis in the liver by TUNEL assay and by immunoblot analysis detecting caspase 3 cleavage confirmed these results and showed increased cell death in p38α/IKK2^{LPC-KO} mice compared tp wild-type or p38α and IKK2 single-mutant mice at 10 h after LPS injection (Fig 8 C, D). In summary, these results indicate that p38α and IKK2 collaborate to protect the liver from LPS/TNF -induced toxicity.

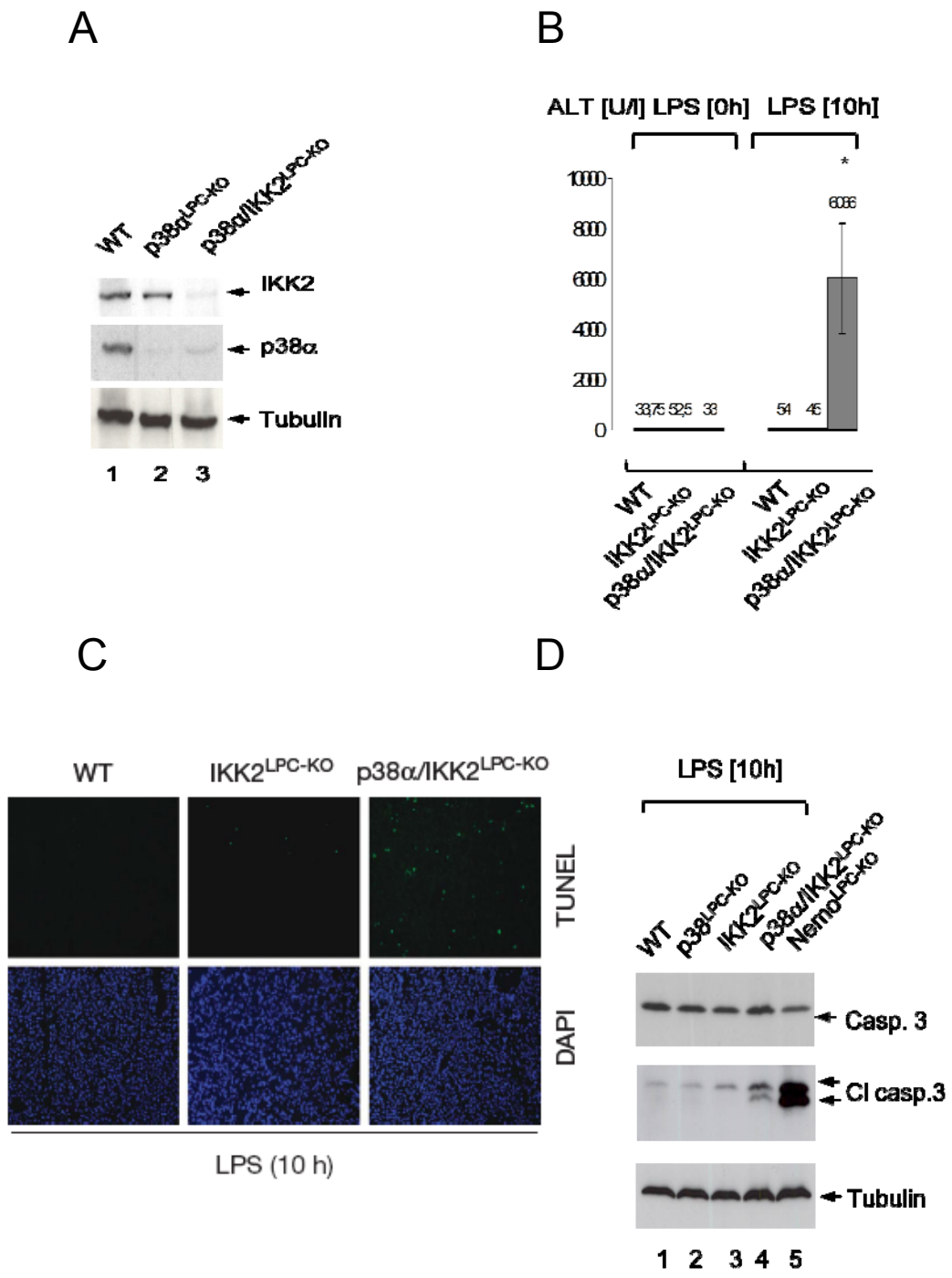

Figure 8: p38α collaborates with IKK2 to protect the liver from LPS-induced toxicity. (A) Immunoblot analysis detecting the expression of IKK2 and p38α in the extracts of liver protein from control, p38α^{LPC-KO} and p38α/IKK2^{LPC-KO} mice. (B) Levels of free circulating ALT were measured in IKK2^{LPC-KO}, p38α/IKK2^{LPC-KO} and control mice before and 10 h after LPS injection. Mean values are depicted above each column. (C) Detection of apoptotic cells by TUNEL assay in liver sections from WT, IKK2^{LPC-KO} and p38α/IKK2^{LPC-KO} mice 10 h after LPS injection. (D) Immunoblot analysis of caspase 3 activation using antibodies that specifically detect total caspase 3 (top panel) or the cleaved form of caspase 3 (middle panel) in liver extracts from mice with the indicated genotypes 10 h after LPS injection. NEMO^{LPC-KO} mice were used as a positive control. Tubulin acts as loading control.

3.2.8 p38α/IKK2^{LPC-KO} mice fail to resynthesize c-FLIP

In order to confirm the inability of p38α/IKK2 negative hepatocytes to activate anti apoptotic NF-κB target genes, the levels of c-FLIP (L) were assessed in the liver of p38α^{LPC-KO}, control and p38α/IKK2^{LPC-KO} mice at various time points after i.p. injection with LPS.

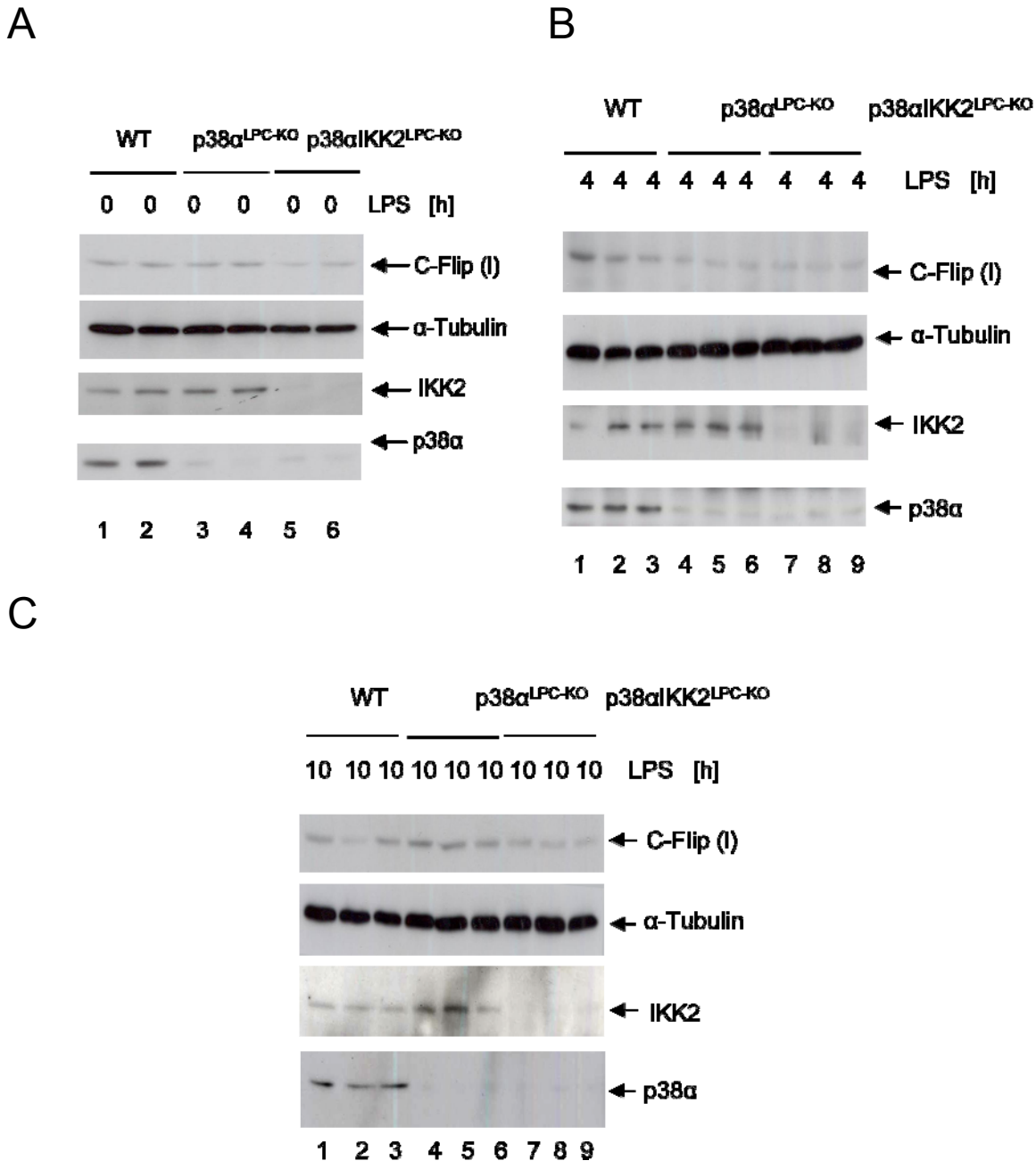

Figure 9: (A-C) The levels of c-FLIP (L) were measured by immunoblot analysis in livers from WT, p38α^{LPC-KO} and p38α/IKK2^{LPC-KO} mice at the indicated time points after LPS injection using c-Flip (L) specific antibodies. Each lane represents an individual animal.

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Immunoblot analysis showed reduced levels of c-FLIP (L) in p38α/IKK2^{LPC-KO} double-mutant mice 10 h after LPS injection compared with single-mutant animals (Fig. 9 C), suggesting that controlling the levels of anti-apoptotic proteins such as c-FLIP (L) is one of the mechanisms through which p38α and IKK2 collaborate to protect the liver from LPS/TNF-induced hepatocyte apoptosis (Fig. 10).

The model:

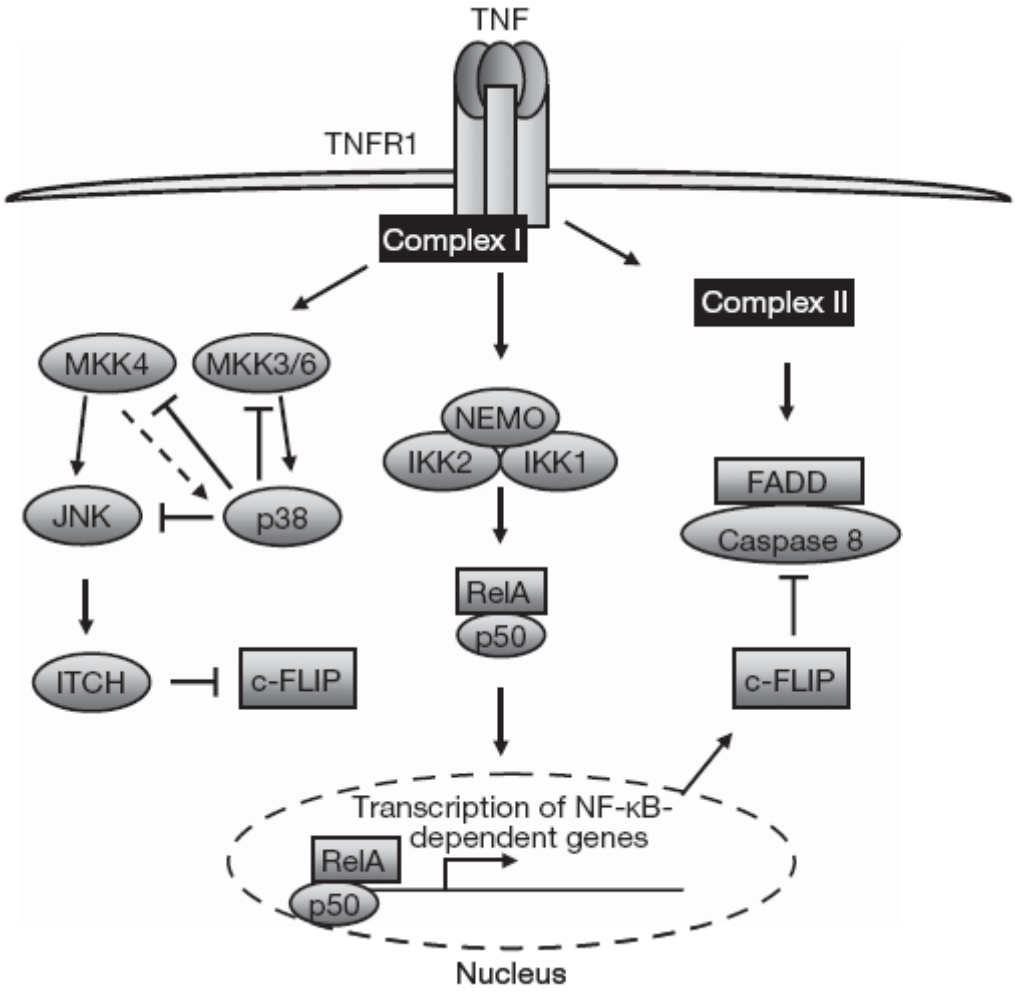


Figure 10: p38α collaborates with IKK2 to prevent liver failure in response to *in vivo* LPS/TNF challenge. TNF binding to TNFRI induces the activation of NF-κB and MAPK pathways, but can also induce cell death through the activation of caspase 8. Activation of NF-κB protects cells from TNF induced cell death by inducing the expression of anti-apoptotic proteins such as c-FLIP. Activation of JNK induces the E3 ubiquitin ligase ITCH to ubiquitinate c-FLIP, leading to its degradation. Lack of p38α in hepatocytes leads to hyperactivation of MKK3/6, MKK4 and JNK, upon *in vivo* LPS challenge. The increased sustained activation of JNK is not sufficient to induce cell death in the p38α deficient liver. When p38α ablation is combined with moderate inhibition of NF-κB, achieved by hepatocyte-restricted IKK2 ablation, *in vivo* LPS challenge results in increased degradation of c-FLIP and liver damage through caspase 8-mediated hepatocyte apoptosis.

3.3 The Role of liver p38α in chronic low grade inflammatory states. A model of high fat diet induced obesity and insulin resistance.

3.3.1 p38α^{LPC-KO} mice do not gain more weight on a high fat diet compared to control mice

To test if liver p38α has a function in chronic low grade inflammatory states we applied a model of diet induced obesity and insulin resistance that is known to induce chronic low grade inflammatory states in various tissues. p38α^{LPC-KO} and wild-type mice were fed a normal chow or a high fat diet (HFD) for 16 weeks. The weight of the mice was monitored on a weekly basis and the average weight of each group was calculated. Neither normal chow diet nor high fat diet induced differences in body weight between p38α^{LPC-KO} and control mice (Fig. 11).

A

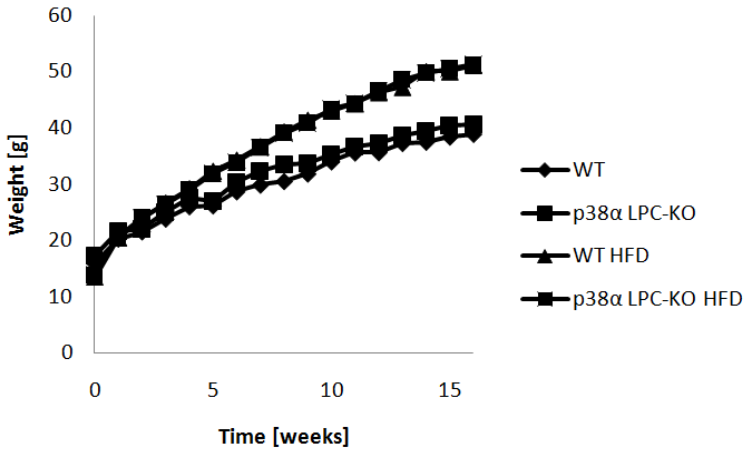


Figure 11: p38α^{LPC-KO} mice do not gain more weight on a high fat diet. (A) After weaning, the average bodyweight of p38α^{LPC-KO} mice and wild-type mice fed chow or high fat diet was determined on a weekly basis for 16 weeks of age (n=25). The average weight of each group was calculated.

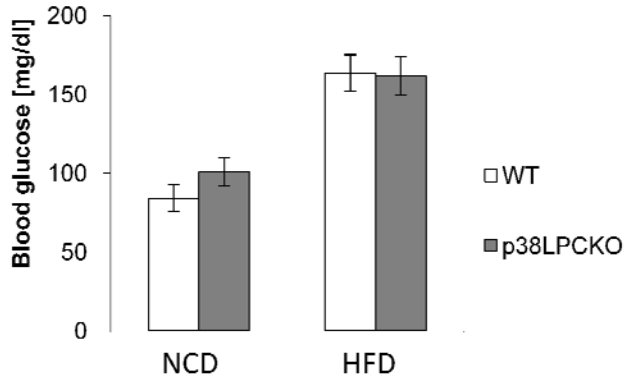
3.3.2 The deletion of p38α in liver parenchymal cell does not lead to an alteration in blood glucose, serum insulin, cholesterol and triacylglyceride levels

The liver is an important organ for glucose metabolism. It responds to insulin by inhibiting hepatic gluconeogenesis and increasing glycogen synthesis. Insulin resistant states lead to hyperglycemia and hyperinsulinemia. Therefore, blood glucose and insulin levels were

measured in p38α^{LPC-KO} and wild-type mice after 16 weeks of chow or HFD, to assess the effect of a liver specific ablation of p38α on glucose (Fig. 12 A) and insulin levels (Fig. 12 B), which are indicators for the degree of insulin resistance. After HFD feeding, animals show hyperglycemia and hyperinsulinemia. Neverthelass, no differences were detected between p38α^{LPC-KO} and wild-type animals.

Moreover, the liver is the metabolic warehouse for lipids in the body. Thus, serum cholesterol (Figure 12 C) and triacyglyceride (Figure 12 D), were measured after 16 weeks of chow or HFD showing no significant differences between p38α^{LPC-KO} and wild-type mice.

A



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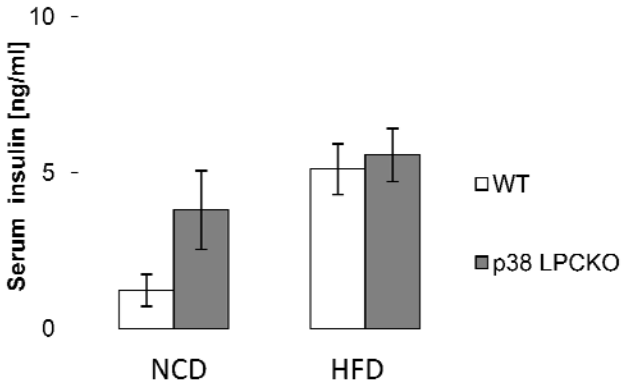
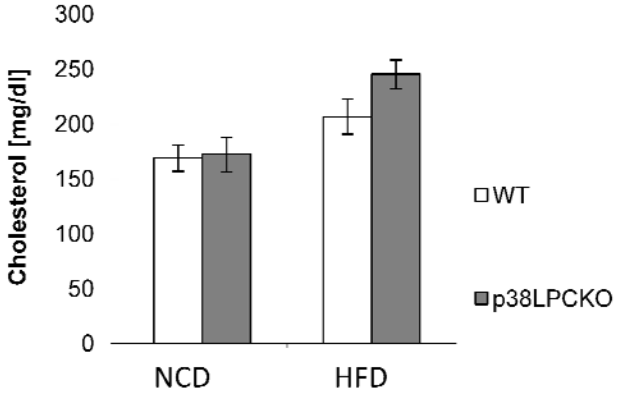


Figure 12: The deletion of p38α in liver parenchymal cell does not lead to an alteration in blood glucose and serum insulin levels (A) Blood glucose levels measured in p38α^{LPC-KO} or control mice after 16 weeks of chow or HFD following an overnight fast. (B) Serum insulin levels from lean and obese p38α^{LPC-KO} and control mice after an overnight fast. n=12

C



D

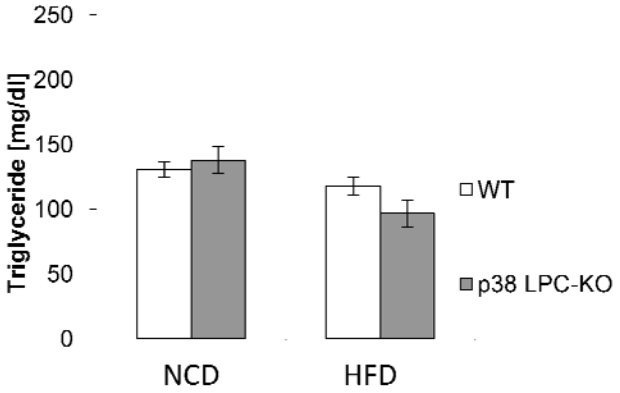


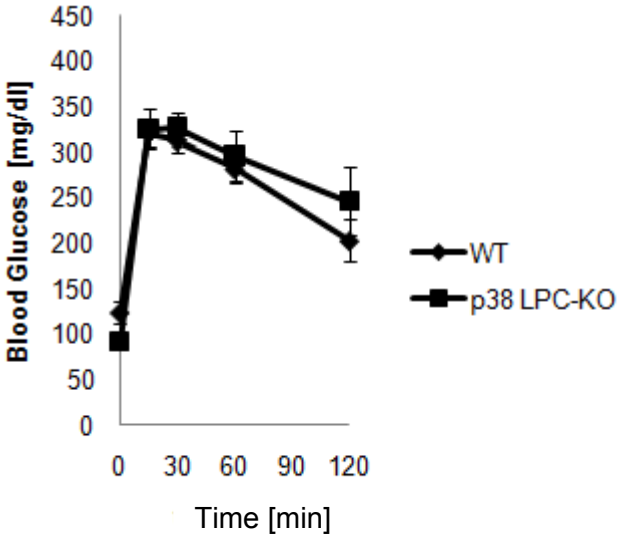
Figure 12: The deletion of p38α in liver parenchymal cell does not lead to an alteration in cholesterol and triacylglyceride levels. Cholesterol (C) and Triacylglyceride (D) levels in the serum of $p38\alpha^{LPC\text{-}KO}$ and control mice, after 16 weeks of chow or HFD. n=12

3.3.3 Obese p38α^{LPC-KO} mice are more insulin resistant than control mice

The liver is a very important organ for glucose homeostasis and responds to insulin by down regulation of hepatic gluconeogenesis and increase of glycogen synthesis. Therefore, the regulation of glucose metabolism in p38α^{LPC-KO} and wild-type mice was adressed by performing glucose (GTT) and insulin tolerance tests (ITT).

p38α^{LPC-KO} and wild-type mice that had been fed a chow or a HFD for 16 weeks were injected with 2g/kg bodyweight glucose into the peritoneal cavity. Blood glucose clearance was assessed 15, 30, 60 and 120 minutes after the injection. While lean p38α^{LPC-KO} mice did not show an altered glucose clearance, compared to wild-type mice (Fig. 13 A), obese p38α^{LPC-KO} mice showed significantly impaired glucose clearance compared to the control animals (Fig 13 B).Thus, obese p38α^{LPC-KO} mice less glucose tolerant than the wild-type animals. To test the response of these animals to insulin, an insulin tolerance test was performed. 0,75 U/kg bodyweight of human regular insulin were injected into the peritoneal cavity of p38α^{LPC-KO} and wild-type mice that had been fed a chow or a HFD for 17 weeks. Figure 13 D shows that obese p38α^{LPC-KO} mice responded much less to the injection of insulin since they showed only a mild decrease in blood glucose levels after insulin injection while the response in wild type animals is significantly stronger. The difference in response to the injected insulin indicates, that obese p38α^{LPC-KO} mice are more insulin resistant than control mice (Fig. 13 D). Lean p38α^{LPC-KO} mice did not show any alterations in insulin sensitivity compared to control mice (Fig. 13 C). In summary, these results clearly indicate that hepatocyte-specific deletion of p38α in obese mice increases insulin resistance.

A



B

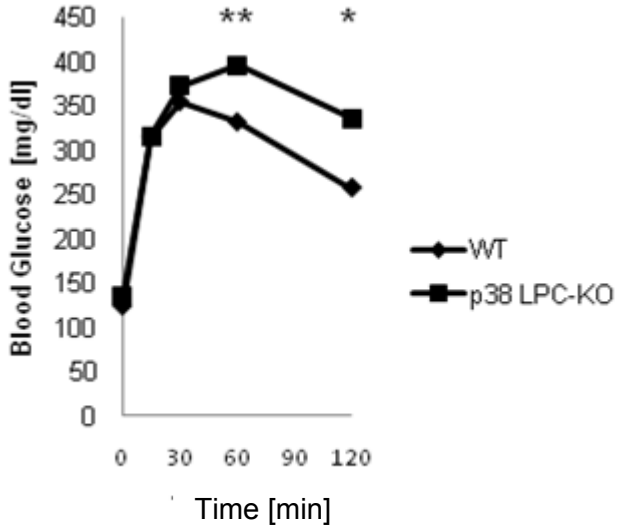
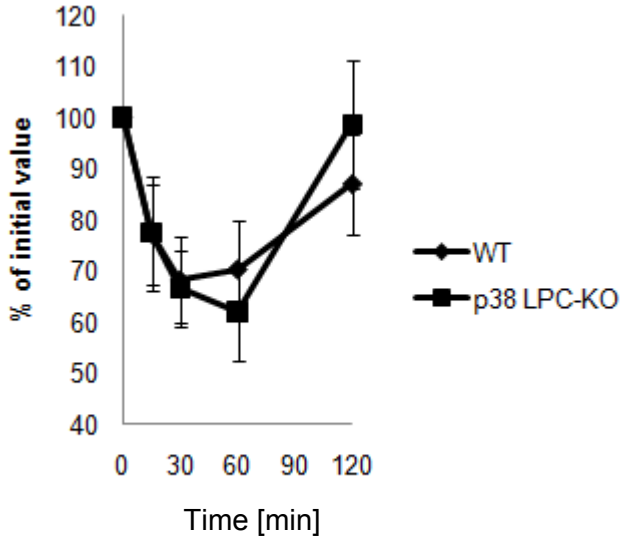


Figure 13: p38α^{LPC-KO} mice are more insulin resistant than control mice: (A) Glucose tolerance tests were performed with lean p38α^{LPC-KO} and control mice at 21 weeks of age. (B) Glucose tolerance tests of mice with the indicated genotypes were performed at 21 weeks of age after 16 weeks of HFD feeding (n=21). Values are mean ± SEM. (Error bars < 1, not visible)

C



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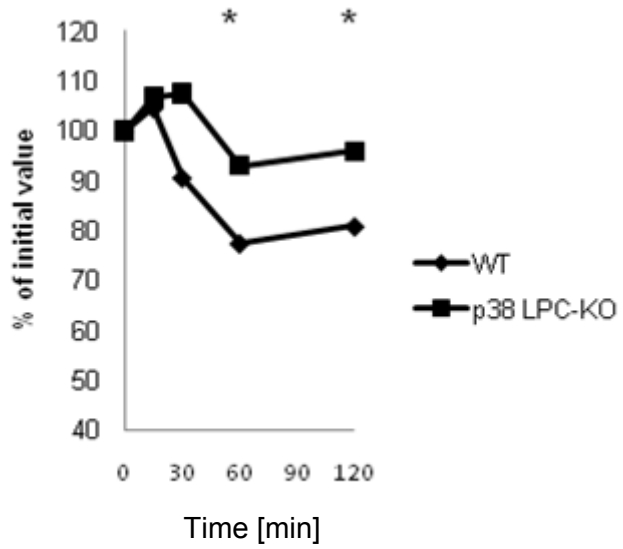


Figure 13: p38α^{LPC-KO} mice are more insulin resistant than control mice (C) Insulin tolerance tests of p38α^{LPC-KO} and control mice were performed at 22 weeks of age after 17 weeks of chow diet feeding. (D) Insulin tolerance tests of mice with the indicated genotypes were performed at 22 weeks of age after 17 weeks of HFD feeding. (n=21) Values are mean ± SEM. (Error bars < 1, not visible)

3.3.4 p38α deficient livers show decreased insulin stimulated signaling

In order to assess insulin mediated signaling in the peripheral organs, obese p38α^{LPC-KO} and wild-type mice were anesthetized and injected with insulin through the *vena cava*.

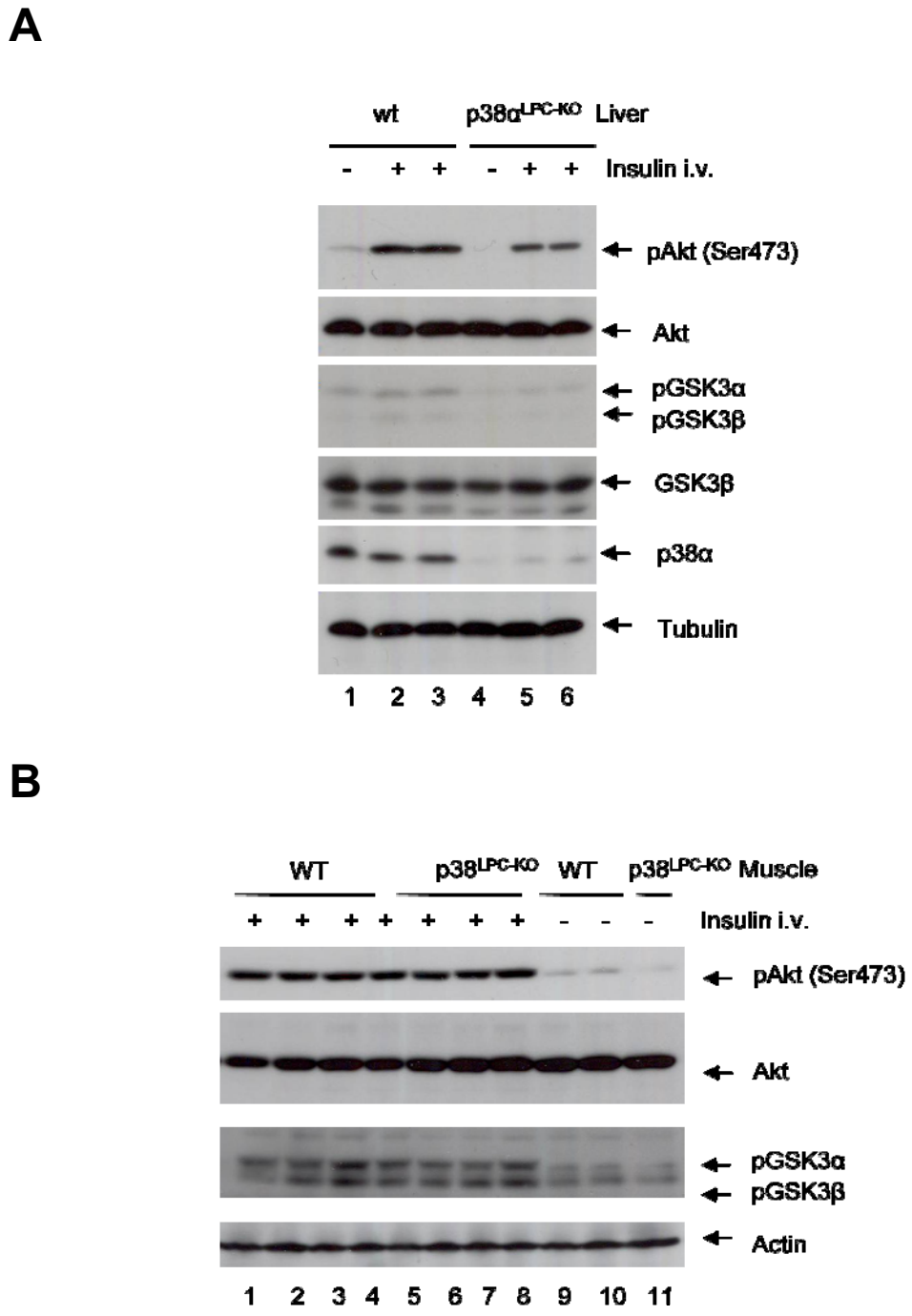


Figure 14 p38α deficient livers show decreased insulin stimulated signaling: p38α^{LPC-KO} mice and control mice that were fed a HFD for 18 weeks were anesthetized and injected with insulin through the *vena cava*. 2 and 5 minutes after the injection livers and muscle were harvested respectively. Insulin signaling in liver (A) and muscle (B) was assessed by immunoblot analysis unsing pASK and pGSK3α/β specific antibodies. Actin/Tubulin serve as loading control.

2 minutes after the injection of insulin the livers were harvested. Muscle was taken 5 minutes after the injection of insulin. Insulin stimulated signal transduction was measured in these organs by immunoblot analysis assessing the phosphorylation status of downstream insulin receptor molecules AKT and GSK3. While insulin stimulated signaling was impaired in the livers of $p38\alpha^{LPC\text{-}KO}$ mice (Fig. 14 A), insulin signaling in muscle taken from $p38\alpha^{LPC\text{-}KO}$ mice was not affected (Fig. 14 B). Thus, the liver specific knockout of p38α results in local insulin resistance in the liver but not in other peripheral organs like the muscle.

3.3.5 p38α deficient primary hepatocytes show decreased insulin stimulated signaling

Next, the insulin stimulated signaling in primary isolated hepatocytes was assessed. $p38\alpha^{LPC\text{-}KO}$ mice and control mice were perfused with collagenase solution to obtain primary hepatocytes. After an overnight culture, control and $p38\alpha^{-/-}$ hepatocytes were stimulated with 200mM insulin at the indicated time points. Immunoblot analysis of the downstream insulin receptor signaling molecule AKT revealed a clear impairment of insulin stimulated signaling in $p38\alpha^{-/-}$ hepatocytes (Fig. 15 A).

A

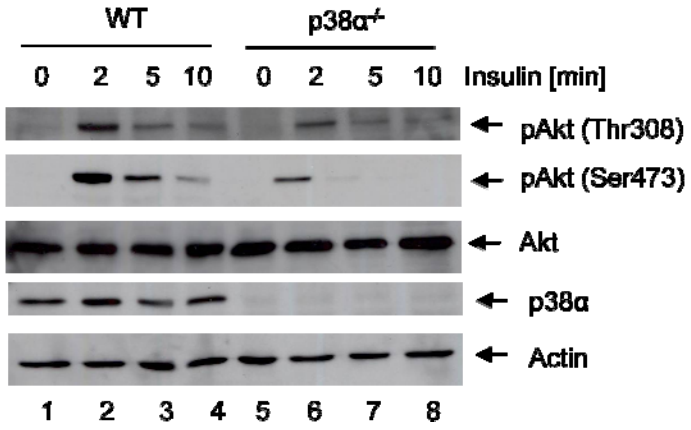


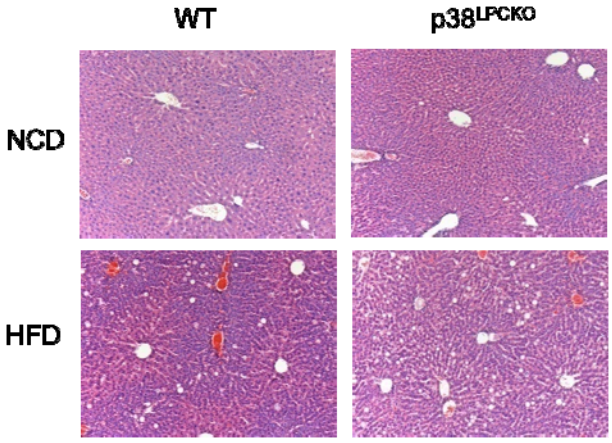
Figure 15: p38α deficient primary hepatocytes show decreased insulin stimulated signaling: Primary isolated hepatocytes were stimulated with 200nM insulin for the indicated times. Assessment of downstream receptor insulin signaling was performed by immunoblot using pAKT specific antibodies. Actin serves as loading control.

The experiments in Figures 14 and 15 unequivocally demonstrate that a deletion of p38α in liver parenchymal cells leads to an impairment of insulin signaling *in vivo* and *in vitro*. Nevertheless, the insulin resistance induced by the knockout of p38α seems to be restricted to the liver, since muscle of $p38\alpha^{LPC\text{-}KO}$ mice shows normal insulin stimulated signaling.

3.3.6 Liver parenchymal cell specific knockout of p38α does not alter the liver lipid content

Impaired insulin signaling can be induced by various mechanisms. Among these causative machanisms are changes in the liver lipid content. In particular diacylglycerides (DAGs) and ceramides have been implicated in the negative regulation of insulin signaling. According to this theory, obesity results in an increased flux of free fatty acids into the circulation and uptake by hepatocytes. Activated fatty acids (i.e., fatty acyl-CoAs) are metabolized primarily via oxidation or storage. When fatty acid flux exceeds the ability of these pathways to dispose of fatty acyl-CoAs, intermediates of fatty acid metabolism (e.g., DAG, PA, LPA, ceramide) accumulate. As a result, these fatty acid intermediates can activate a number of different serine kinases that negatively regulate insulin action. Ceramide has also been shown to impair insulin action, although the precise mechanisms leading to insulin resistance are, to date, unknown (Summers 2006).
In order to investigate a possible shift in lipid liver content which might be causal to the impaired insulin signaling in p38α deficient livers and hepatocytes, liver sections were analyzed. H&E and oil red O stainings were performed. H&E staining did not show alterations in liver histology between p38α deficient and wild-type livers (Fig. 16 A). Also the lipid content did not seem to vary indicated between p38α deficient and wild-type livers as indicated by the oil red O staining (Fig. 16 B).

A



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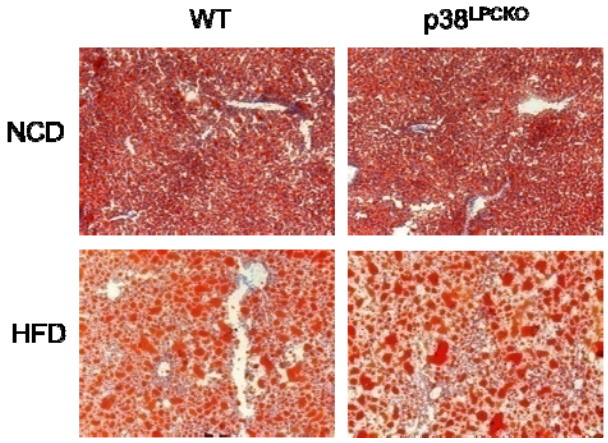
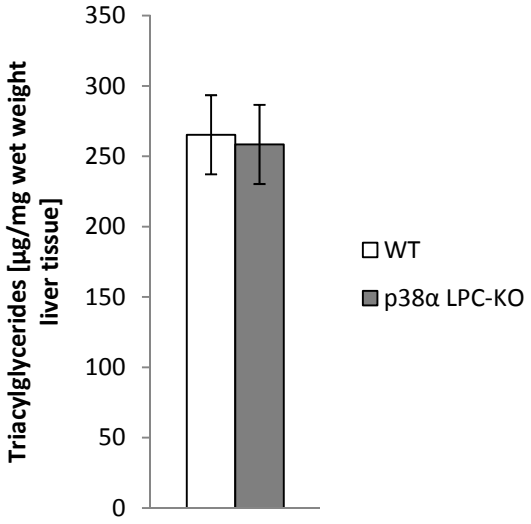


Figure16: The liver parenchymal knockout of p38α does not alter the liver lipid content: (A) Representative pictures of Haematoxylen and Eosin staining on liver sections, taken from normal chow or HFD diet treated $p38\alpha^{LPC\text{-}KO}$ or wild-type mice. (B) Liver lipid content in $p38\alpha^{LPC\text{-}KO}$ or wild-type mice after chow or HFD stained by oil red O.

A qualitative lipid analysis of different lipid subclasses by thin layer chromatography was performed to reveal a possible alteration in different lipid classes between p38α deficient and wild-type livers. Nevertheless, after 18 weeks chow or HFD feeding the amounts of triacylglycerides, cholesterol esters, 1,2 DAGs, 1,3 DAGs and free fatty acids did not show significant differences (Fig. 16 C and D). Therefore, a change in lipid quantity and quality can be ruled out as a causative factor for the impaired insulin signaling in p38α deficient livers and hepatocytes.

C



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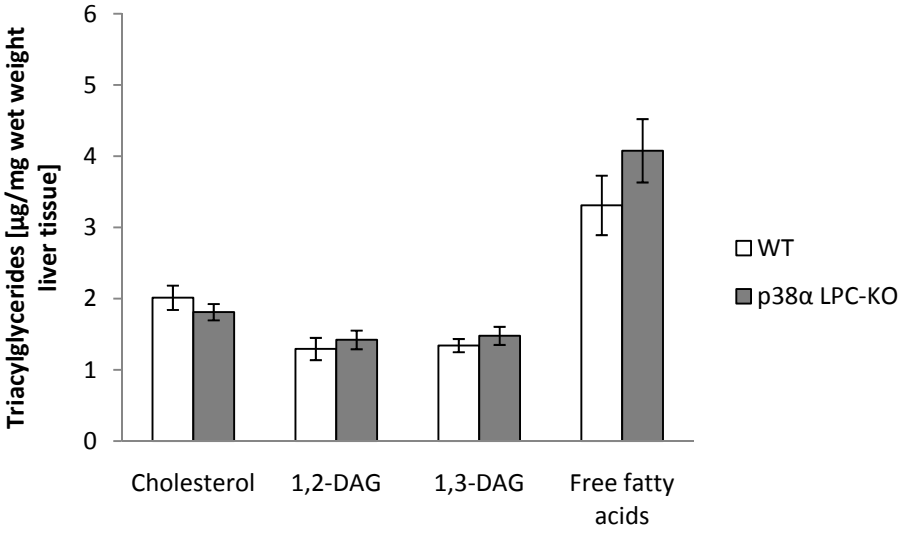
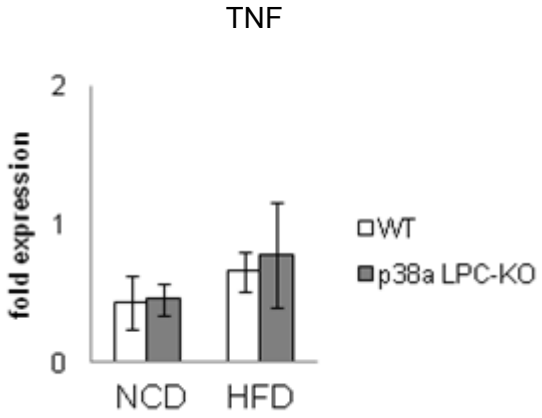


Figure 16: Unaltered liver lipid content in p38α deficient livers: (A) Assessment of triacylglycerides, (B) cholesterol esters, 1,2 DAGs, 1,3 DAGs and free fatty acids by thin layer chromatography from p38α or control livers after 18 weeks of HFD. n=12

3.3.7 The deletion of p38α in liver parenchymal cells does not alter the expression of TNF, IL-6 and IL1-β in liver

Increased expression of inflammatory cytokines has been reported to be a cause for the development of insulin resistance (Hotamisligil 2006). In order to assess the expression levels of inflammatory cytokines in the liver, quantitative PCR was performed on cDNA isolated from p38α deficient and wild-type livers taken from mice that received chow or HFD for 18 weeks.

A



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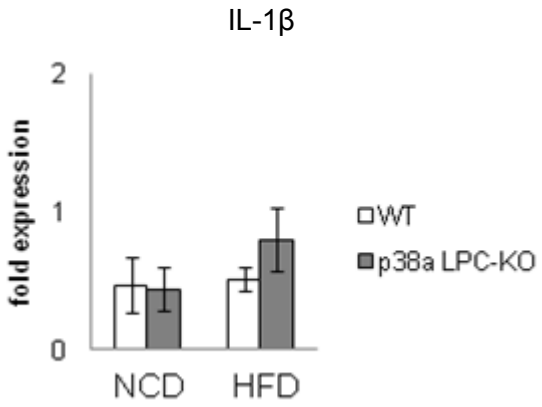


Figure 17: Unaltered expression of inflammatory cytokines in liver. Quantitative PCR performed on RNA isolated from p38α deficient and control livers, taken from mice that were fed a chow or a high fat diet. Expression levels of (A) TNF, (B) expression levels of IL-1β. n=5

C

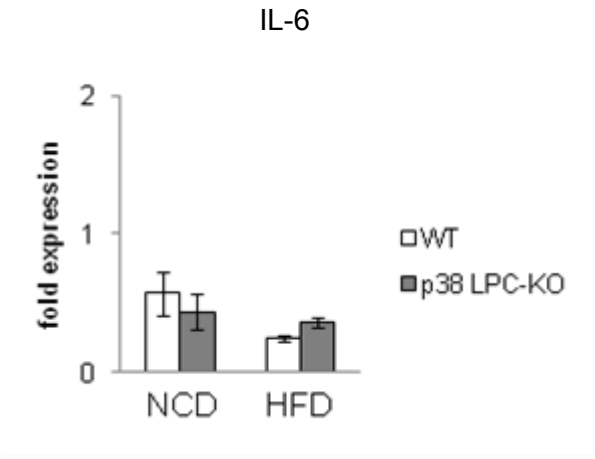


Figure 17: Unaltered expression of inflammatory cytokines in liver. Quantitative PCR performed on RNA isolated from p38α deficient and control livers taken from mice that were fed a chow or a high fat diet. (C) Expression levels of IL-6. n=5

TNF, IL1-β and IL-6 are key cytokines that have been reported to induce insulin resistance in various organs (Hotamisligil 2006). Nevertheless, p38α deficient livers did not show an altered expression of TNF, IL-6 and IL-1β (Fig. 17 A-C). This suggests that the increase in insulin resistance in obese p38α^{LPC-KO} mice is not caused by an increased expression of inflammatory cytokines in the liver.

3.3.8 Unaltered expression of inflammatory cytokines in adipose tissue taken from p38α^{LPC-KO} and control mice.

Independently from the unaltered cytokine expression in the liver, factors that are released by the liver could lead to a change in cytokine production in other tissues like the adipose tissue. Therefore, the expression profile of inflammatory cytokines in the adipose tissue was investigated. Similar to the liver, there were no changes in the expression of TNF (Fig. 18 A), IL-6 (Fig. 18 B), IL-1 β (Fig. 18 C) and MCP-1 (Fig. 18 D) between tissues taken from p38α^{LPC-KO} mice and wild-type mice. Nevertheless, there was a 3 fold elevation in expression of TNF in response to the HFD treatment in tissues taken from p38α^{LPC-KO} mice and control mice (Fig. 18 A).

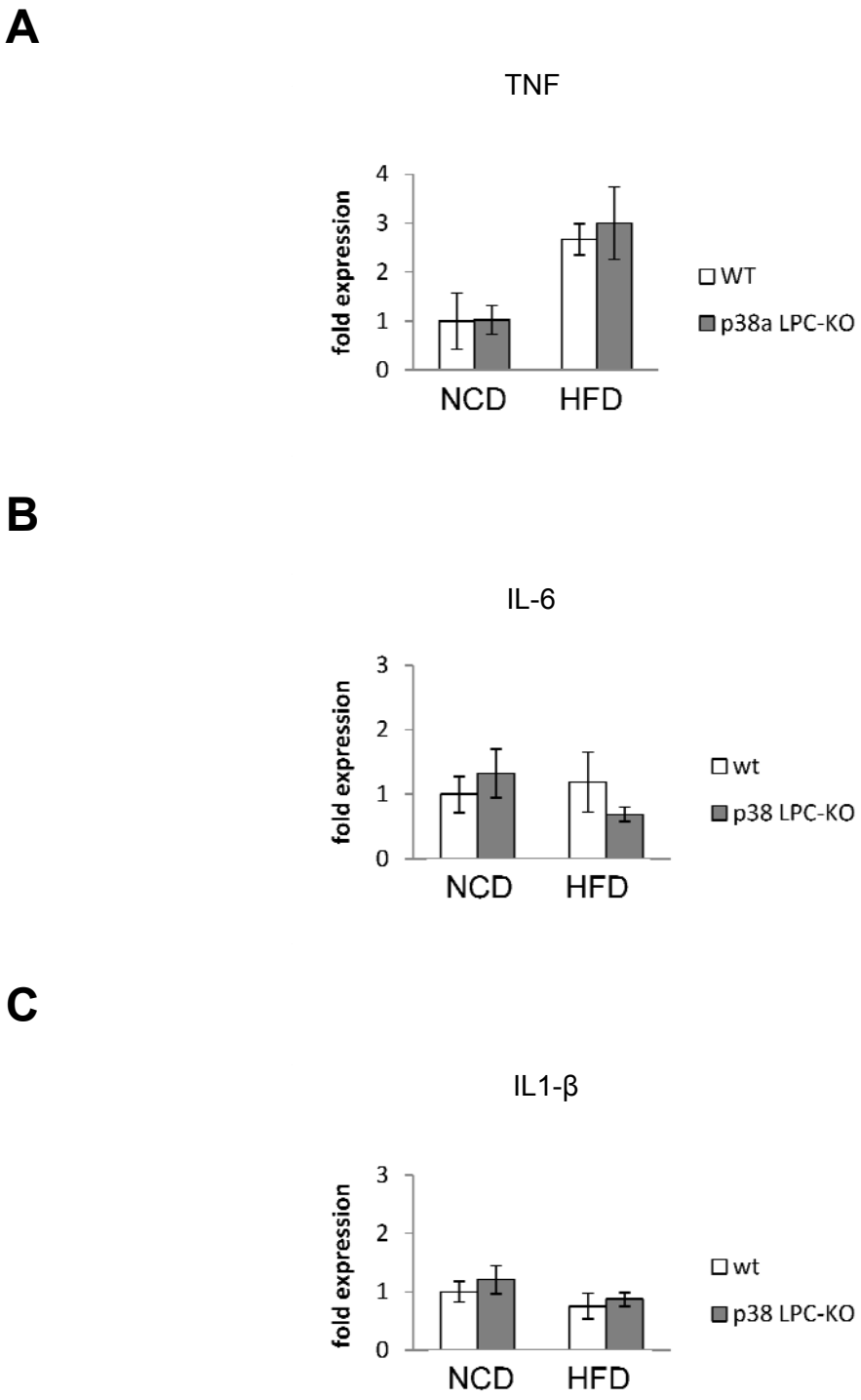


Figure 18: Unaltered expression of inflammatory cytokines in the adipose tissue. Quantitative PCR performed on RNA isolated from adipose tissue isolated from p38α^{LPC-KO} and control mice that were fed a chow or a high fat diet. Expression levels of (A) TNF, (B) IL6, and (C) IL1-β.n=5

D

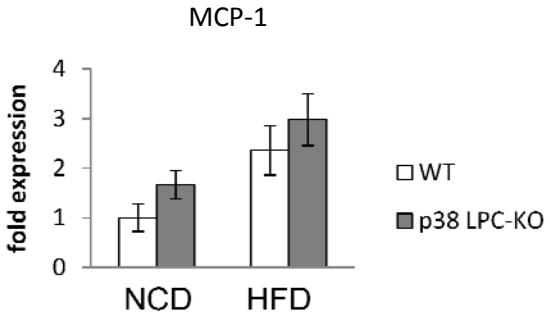


Figure 18: Unaltered expression of inflammatory cytokines in the adipose tissue. Quantitative PCR performed on RNA isolated from adipose tissue isolated from p38α^{LPC-KO} and control mice that were fed a chow or a high fat diet. Expression levels of (D) MCP-1.

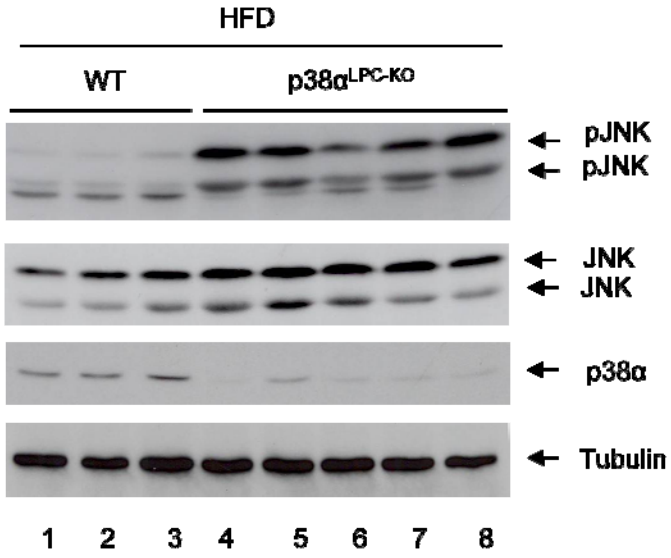
3.3.9 Increased activation of JNK in the livers of obese p38α^{LPC-KO} mice

In the LPS/TNF induced liver toxicity model it became apparent that p38α deficient hepatocytes show hyperactivated JNK in response to TNF (Heinrichsdorff, Luedde et al. 2008). Therefore, the activation of JNK in livers taken from p38α^{LPC-KO} mice and control mice that were fed a HFD for 18 weeks was assessed. Consistent with the data from the acute liver toxicity model, livers taken from obese p38α^{LPC-KO} mice had a higher activation of JNK than livers taken from wild-type mice (Fig. 19 A).

Hyperactivation of JNK has been shown in various models of obesity and insulin resistance where it was reported to have an inhibitory effect on insulin signaling. According to these models, JNK is able to interact with and phosphorylate the insulin receptor substrate 1 (IRS1) at a negatively regulatory serine position (Hirosumi, Tuncman et al. 2002; Solinas, Naugler et al. 2006; Solinas, Vilcu et al. 2007).

In addition to livers taken from HFD treated p38α^{LPC-KO} mice, isolated resting primary hepatocytes were tested for JNK activity. Elevated levels of JNK activity were detected independent from a stimulus (Fig. 19 B), suggesting that the p38α knockout results in intrinsic JNK hyperactivation.

A



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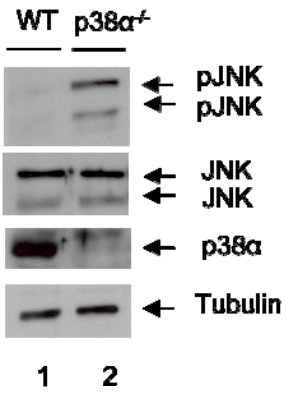

Figure 19: Hyperactivation of JNK in p38α^{LPC-KO} livers and primary hepatocytes. (A) Livers taken from obese p38α^{LPC-KO} and control mice were tested for JNK activity by immunoblot. Phospho-specific JNK antibodies were used to detect JNK activity. (B) Primary p38α deficient and control hepatocytes were tested for JNK activity using immunoblot analysis. Phospho-speciffic JNK antibodies were used to detect JNK activity. Tubulin serves as a loading control.

3.3.10 Inhibition of JNK can only partially restore insulin signaling in p38α deficient hepatocytes

JNK inhibitors are commonly used *in vitro* to impair JNK signaling and have been discussed as a potential therapeutic for various diseases including insulin resistance and type 2 diabetes. We stimulated primary p38α deficient and control hepatocytes with insulin in the presence or absence of the JNK inhibitor VII. Even though the activity of JNK was effectively reduced in the presence of the inhibitor, which is indicated by the decrease in phosphorylation of the JNK substrate c-Jun, insulin signaling indicated by AKT phosphorylation, was only mildly restored (Fig. 20).This result indicates that JNK hyperactivation might not be the only causative mechanism that leads to the impairment of insulin signaling in p38α deficient cells.

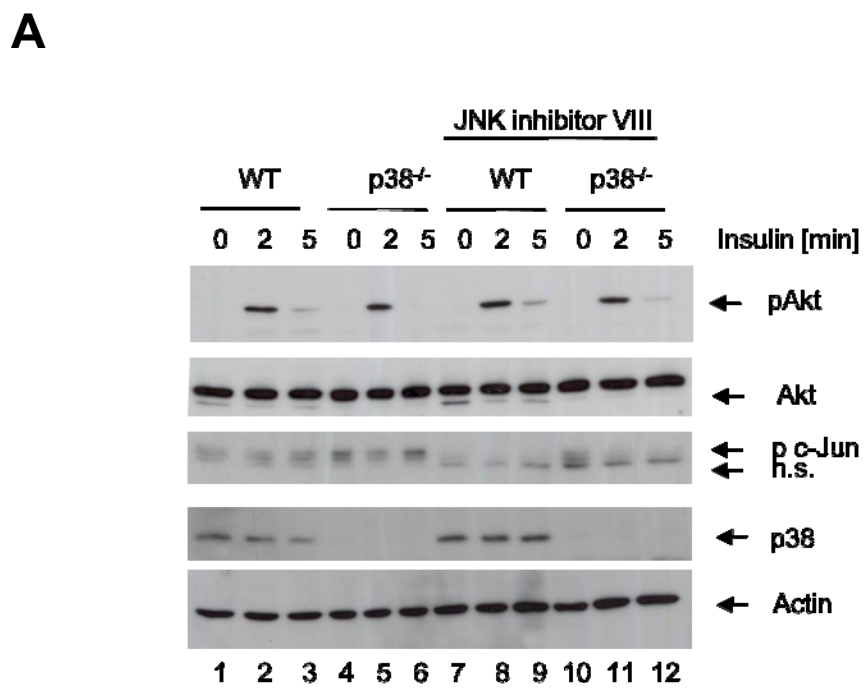

Figure 20: Inhibition of JNK can only partially restore insulin signaling in p38α deficient hepatocytes. p38α and control hepatocytes were stimulated with 200nM insulin in the presence or absence of 10nM JNK inhibitor. Insulin induced signaling was assessed by immunolot analysis using phospho-AKT specific antibodies. The effect of the JNK inhibition was tested using phosphor-c-Jun specific antibodies. Actin serves as a loading control.

3.3.11 Increased phosphorylation of Ser307 in p38α deficient hepatocytes

One of the molecular mechanisms that were shown to impair insulin signaling is the phosphorylation of the insulin receptor substrate (IRS) at negative regulatory serine positions that impair the interaction with the insulin receptor substrate and the next molecule in the insulin signaling pathway, the PI3 kinases. Various serine/threonine kinases have been implicated in the negative regulatory phosphorylation of the insulin receptor substrate.

A

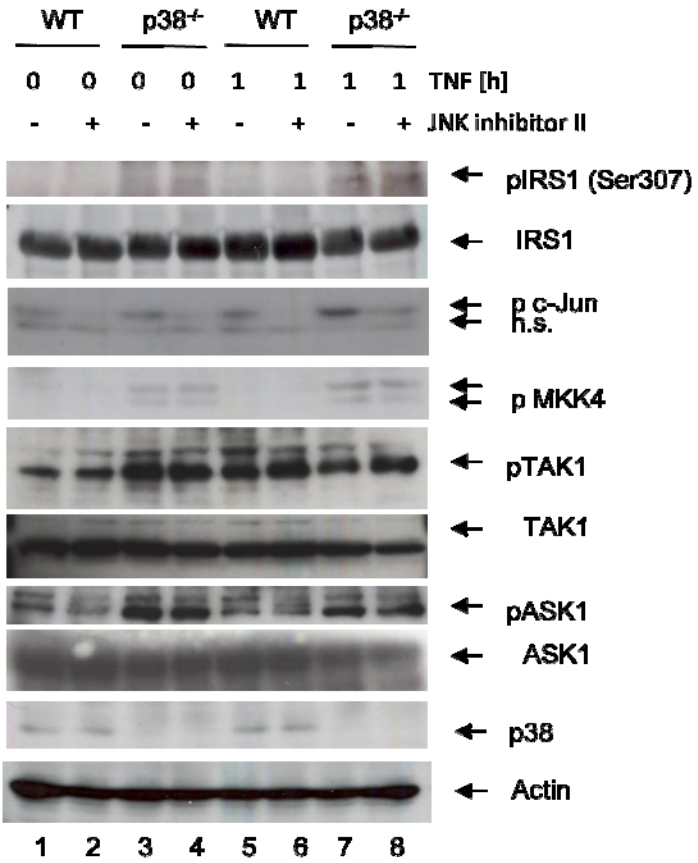

Figure 21: p38α deficient hepatocytes show increased phosphorylation of IRS1 at serine 307. Primary isolated p38α defecient and control hepatocytes were stimulated with TNF for 1 hour. Immunoblot analysis was performed to assess the phosphorylation of IRS1 at serine 307. Actin serves as a loading control.

Phosphorylation of the insulin receptor substrate at a negative regulatory serine position 307 was tested in p38α and wild-type hepatocytes that were stimulated with TNF for 1 hour, in the presence or absence of a JNK inhibitor. Immunoblot analysis showed that p38α deficient hepatocytes have increased serine 307 phosphorylation of the IRS1, before and after

stimulation with TNF. Interestingly, the presence of a JNK inhibitor did not impair this degree of phosphorylation suggesting that other kinases are also involved in the phosphorylation of the IRS1. Furthermore, the activation of upstream JNK kinases MKK4, which is an MAP2K, as well as that of ASK and TAK1, which are MAP3Ks was tested (Fig. 21). Surprisingly, immunoblot analysis showed that several upstream kinases of JNK including MKK4, ASK1 and TAK1 are hyperphosphorylated in p38α deficient hepatocytes, independent from TNF stimulation.

3.3.12 p38α negatively regulates JNK and upstream kinases through an unknown mechanism and therefore positively regulates insulin signaling

In summary p38α seems to control the activation status of various serine/threonine kinases involved in JNK signaling. Besides hyperativated JNK, MKK4, TAK1 and ASK1 are also hyperactivated in p38α deficient cells. Together, these kinases might lead to a phosphorylation of the IRS at Serine 307 which prevents the transduction of the insulin signal. As a result, hepatic gluconeogenesis is not inhibited by an insulin stimulus and glycogen synthesis is not increased.

The model:

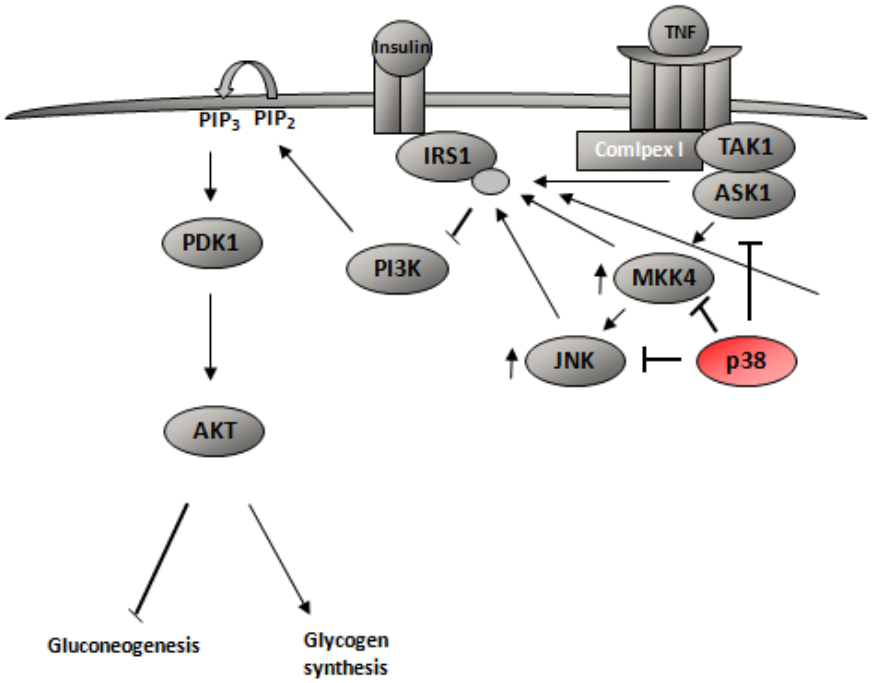

Fig. 22: p38α negatively regulates JNK and upstream kinases through an unknown mechanism and therefore positively regulates insulin signaling.

4. Discussion

The role of liver p38α in acute and chronic inflammatory reactions

p38α has been identified as an important player in inflammatory reactions in numerous studies (Guan, Buckman et al. 1998; Johnson and Bailey 2003; Hollenbach, Neumann et al. 2004). As the central part of this thesis the function of p38α in acute and chronic inflammatory conditions in the liver was examined. In order to do this, a conditional mouse model was utilized taking advantage of the Cre-loxP recombination system to generate a liver parenchymal specific knockout of p38α (Heinrichsdorff, Luedde et al. 2008).

4.1 The role of liver p38α in acute inflammatory reactions

4.1.1 Despite hyperactivation of JNK, p38α^{LPC-KO} mice are not sensitive to LPS-induced liver failure

Acute liver damage can be caused by a wide range of stimuli. TNF has been shown to play an outstanding role during the development of liver failure and is known to activate MAP kinases including p38α. In order to find new targets for medical intervention in cases of acute liver failure, it is important to gain more knowledge about the molecular events that precede this severe medical condition. LPS is one of the most potent TNF inducers but usually does not cause hepatocyte apoptosis unless it is co-administered with D-Galactasoamine (GalN) which specifically blocks gene transcription in the liver (Decker and Keppler 1974; Bradham, Plumpe et al. 1998). The aim of the first part of this thesis was to analyze the LPS/TNF induced apoptotic signals in mice specifically lacking p38α in liver parenchymal cells. To study the role of p38α in acute inflammatory liver conditions in a more physiological context we did not co-administere GalN with the LPS.

Upon LPS injection, deletion of p38α in liver parenchymal cells does not sensitive mice to hepatocyte apoptosis (Fig. 2). This result was somewhat surprising since the injection of LPS caused massive hyperactivation of JNK in the livers of these mice (Fig 3 A). In various studies JNK has been described to have a proapoptotic role (Davis 2000; Weston and Davis 2002). In the liver, it was shown that LPS injection causes hepatocyte apoptosis through the proteasomal degradation of c-FLIP (L) when anitapoptotic signals are inhibited through the injection of GalN. (Chang, Kamata et al. 2006). Furthermore, upon liver parenchymal cell specific deletion of NEMO, which prevents canonical NF-κB signaling in hepatocytes, LPS injection caused massive liver apoptosis. Interestingly, after injection of LPS, the activation of

JNK seems to be weaker in NEMO $^{LPC-KO}$ livers than in p38α^{LPC-KO} livers (Fig. 3 A, B). (Luedde, Beraza et al. 2007). Compared to control mice the activation of JNK in NEMO $^{LPC-KO}$ livers is moderately prolonged (Fig. 3 B). These results indicate that p38α negatively regulates JNK. In livers that have intact NF-κB signaling, hyperactivation of JNK is not sufficient to induce hepatocyte death. Conversely, abolishment of NF-κB signaling in the NEMO^{LPC-KO} mice hypersensitizes hepatocytes to LPS/TNF induced cell death, without leading to elevated levels of JNK. Thus, in a model of LPS/TNF induced liver toxcicty, NF-κB signaling seems to play an important role in suppressing a proapoptotic stimulus executed by JNK, leading to the survival of hepatocytes. Mechanistically, NF-κB signaling seems to be responsible for the expression of various antiapoptotic genes including c-FLIP, which competes with FADD for the binding to procaspase 8 and therefore inhibits the apoptotic potential of the TNF receptor complex II (Micheau, Lens et al. 2001; Chang, Kamata et al. 2006). Immunoblot analysis performed on livers taken from p38α^{LPC-KO}, NEMO^{LPC-KO} and control mice after LPS injection, indicated a slight decrease of c-FLIP (L) protein levels 4 h after LPS injection (Fig. 5 A). However, 10 h after LPS injection, the levels of c-FLIP (L) were similar in p38α^{LPC-KO} and control mice (Fig. 2 A). By contrast, the levels of c-FLIP (L) were diminished in NEMO deficient livers 10 h after LPS injection (Fig. 5 A, lanes 7–9), which is consistent with the increased hepatocyte apoptosis detected at this point. These results indicate that even a massive activation of JNK is not sufficient to reduce intracellular levels of c-FLIP (L) below the threshold required to sensitize hepatocytes to LPS/TNF-induced death, while even a moderate prolongation of JNK activity results in degradation of c-FLIP (L) and apoptosis of hepatocytes with abolished NF-κB signaling.

4.1.2 Oxidative stress and MAP kinase phosphatases are not involved in the hyperactivation of JNK in p38α deficient livers.

There is accumulating evidence that p38α generally suppresses JNK activity independent of cell type. Besides the finding that JNK is hyperactivated in p38α defecient hepatocytes, it was also shown in other cell types that JNK is negatively regulated by p38α (Hui, Bakiri et al. 2007; Perdiguero, Ruiz-Bonilla et al. 2007; Sakurai, He et al. 2008).

Several reasons could account for the hyperactivation of JNK in p38α deficient hepatocytes: Elevated oxidative stress could be the culprit leading to the hyperactivation of JNK in the absence of p38α (Kamata, Honda et al. 2005). In order to test this hypothesis, animals were fed the antioxidant compound butylated hydroxyanisole (BHA) for 48 hours, before the injection of LPS. Nevertheless, despite treatment with BHA, p38α deficient livers still showed

hyperactivation of JNK after LPS injection ruling out oxidative stress as a causative mechanism for JNK hyperactivation in p38α deficient livers (Fig. 6 A).

The p38 pathway also regulates the activity of various transcription factors (Hazzalin, Cano et al. 1996; Wang and Ron 1996; Han, Jiang et al. 1997; Janknecht and Hunter 1997; Whitmarsh, Yang et al. 1997; Huang, Ma et al. 1999; Zhao, New et al. 1999; Yee, Paulson et al. 2004). Thus, p38α might be involved in the expression of phosphatases controling JNK activity. To test this hypothesis, protein levels of various MAP kinase phosphatases were assessed. MKP1, MKP2 and MKP3 that are known to be involved in the regulation of JNK activity (Keyse 2008) and were shown to dephosphorylate MAP kinases at both phosphorylation sites, the Thr and Tyr residue in the activation loop effectively abrogating the activity of MAP kinases. Therefore, they are called dual specifity phosphatases (DUSPs) (Liu, Shepherd et al. 2007). Furthermore, protein levels of pp5 (Golden, Swingle et al. 2008), pp1me pp1a and pp2a (Junttila, Li et al. 2008) were examined, which are phosphatases generally involved in cellular stress responses. As shown in Figure 6, none of the potential phosphatases regulating JNK showed altered expression in p38α deficient livers ruling out the possibility that altered protein levels of JNK phosphatases are involved in the hyperactivation of JNK (Fig. 6 B and C). In addition to transcriptional regulation, MKPs can also be regulated by and protein stabilization and phosporytation which inceases catalytic activity. This study does not rule out that the possibility that p38 is involved in phosphorylation of MKPs or stabilization of MKP transcripts. Therefore, a possible effect of p38α on the activity of phosphatases has to be studied in further detail.

In addition, upstream kinases of JNK are also hyperactivated in the livers of $p38\alpha^{LPC\text{-}KO}$ mice upon LPS injection. The phosphorylation of JNK is controlled by two upstream kinases, MAP kinase kinase (MKK) 7 and 4. MKK7 was reported to specifically activate JNK and seems to be activated by TNF and environmental stress (Hui, Bakiri et al. 2007). Surprisingly, immunoblot analysis showed increased phosphorylation of MKK4, but not MKK7, in the liver of $p38\alpha^{LPC\text{-}KO}$ mice compared to wild-type livers, after LPS injection (Fig. 7 A). Furthermore, increased phosphorylation levels of the p38α activating kinases MKK3/6 in the livers of LPS-injected $p38\alpha^{LPC\text{-}KO}$ mice were found (Fig. 7 B). The finding that MKK4 is hyperactivated in livers of $p38\alpha^{LPC\text{-}KO}$ mice after LPS injection is surprising, since it was reported to phosphorylate both JNK and p38 MAPK, but has never been shown to be activated by TNF. (Derijard, Raingeaud et al. 1995; Minden, Lin et al. 1995; Moriguchi, Toyoshima et al. 1997; Tournier, Whitmarsh et al. 1999). Therefore, in the liver MKK4 can also be accounted as a kinase that is able to activate JNK, in response to TNF. The fact that hyperactivation of MKK7, a well accepted MAP2K in TNF signaling, was not detected might be due to the lack of a good commercial antibody to detect the phosphorylated form of MKK7. There are also

indications that upstream kinases of MKK4, including MLK3, are hyperactivated in the livers of p38α^{LPC-KO} mice after LPS injection. (Fig. 7 C).

Taken together these results suggest that neither oxidative stress nor JNK phosphatases seem to regulate the hyperactivation of JNK. On the other hand, the hyperactivation of the upstream kinases MKK4, MKK3/6 as well as the MAP3K MLK3 suggest a defect more proximal to the TNFR I. One possible explanation that has not been investigated thus far could be an influence of p38α on the scaffolding of adaptor proteins like JIP, where JNK and also MKKs scaffold to increase specificity and intensity of the signal (Yasuda, Whitmarsh et al. 1999; Chang and Karin 2001). Furthermore, a defect in negative regulation of signal transduction at the receptor which seems to rely on the dissociation of a multiprotein signaling complex controlled by the ubiquitin mediated degradation of TRAF3 (Matsuzawa, Tseng et al. 2008; Karin and Gallagher 2009) could be causal for hyperactivation of JNK. Even though this mechanism is mainly described for the TNFR family member CD40, there is evidence that it may also apply to the TNFR itself (Matsuzawa, Tseng et al. 2008). The possibility that p38α might have a regulatory function in this complex mechanism, has to be investigated in further detail.

4.1.3 p38α and IKK2 collaborate to prevent LPS/TNF induced liver failure

Despite massive hyperactivation of JNK, p38α^{LPC-KO} livers are not sensitized to endotoxin induced cell death. Thus, it is quite likely that survival pathways keep hepatocytes in a state that does not allow the induction of apoptosis. To this end, the NF-κB signaling pathway seems to play an important role. Hepatocytes deficient for canonical NF-κB signaling are hypersensitive to LPS/TNF induced apoptosis (Fig. 2 B and C) (Luedde, Beraza et al. 2007). Partial inhibition of NF-κB signaling through deletion of IKK2 is not sufficient to induce hepatocyte death upon *in vivo* LPS injection (Fig. 8 A) (Luedde, Heinrichsdorff et al. 2008). Nevertheless, it was hypothesized that partial inhibition of NF-κB together with hyperactivation of proapoptotic JNK signaling should sensitize p38α/IKK2 deficient hepatocytes towards LPS/TNF induced cell death. Indeed, elevated serum transaminases, TUNEL staining and caspase 3 cleavage after LPS injection show that p38α/IKK2^{LPC-KO} mice are sensitized to LPS/TNF induced hepatocyte death. Thus, in a model of LPS/TNF induced liver toxicity, partial impairment of NF-κB signaling, in concert with hyperactivation of JNK, seems to bring hepatocytes into a state that allows the induction of apoptosis. This experiment highlights the fine balance between hepatocyte survival and death that is mainly influenced by the balance of pathways that are activated downstream of TNFR I namely NF-κB, JNK and p38α.

In order to dissect the molecular mechanisms that are responsible for the sensitization of p38α/IKK2^{LPC-KO} mice towards LPS/TNF induced hepatocyte death, anti-apoptotic target genes induced by NF-κB were examined. NF-κB can promote the expression of several antiapoptotic genes such as TRAF1, TRAF2, cIAP-1, c-IAP-2, and c-FLIP (Wang, Mayo et al. 1998). One of the NF-κB target genes that is involved in the prevention of TNF induced apoptosis is c-FLIP (Wang, Mayo et al. 1998; Kreuz, Siegmund et al. 2001; Micheau, Lens et al. 2001). The short form of c-FLIP, c-FLIP (s), contains two death effector domains and is structurally related to the viral FLIP inhibitors of apoptosis, whereas the long form, c-FLIP (L), contains an additional caspase-like domain, in which the active-centre cysteine residue is substituted by a tyrosine residue. c-FLIP (s) and c-FLIP (L) interact with the adaptor protein FADD and the protease FLICE and potently inhibit apoptosis induced by all known human death receptors, including TNFR I (Irmler, Thome et al. 1997). It has also been shown that c-FLIP (L) plays a crutial role in JNK mediated cell death, since TNF mediated JNK activation accelerates turnover of the NF-κB induced antiapoptotic protein c-FLIP by phosphorylation and activation of the E3 ubiquitin ligase Itch. This in turn specifically ubiquitinates c-FLIP and induces its proteasomal degradation (Chang, Kamata et al. 2006)

Figure 6 clearly shows that c-FLIP (L) levels decrease in the livers of p38α^{LPC-KO} mice, 4 hours after the injection of LPS. 10 hours after the injection of LPS, c-FLIP (L) protein is resythesized preventing induction of death receptor mediated apoptosis. In contrast to this, after LPS injection, p38α/IKK2 deficient livers fail to resynthesize c-FLIP (L). c-FLIP levels remain below the threshold, allowing hepatocyte apoptosis to take place. Thus, the balance between JNK and NF-κB activation plays a crucial role deciding over hepatocyte survival and death in LPS/TNF challenged livers that show acute inflammatory reactions. The NF-κB target gene c-FLIP, which can be degraded through JNK by the activation of the E3 ubiquitin ligase ITCH seems to play a crucial role protecting the liver from massive hepatocyte death, in states where JNK activation is high. Therefore, p38α and IKK2 collaborate to prevent liver failure.

In conclusion, these results have important implications for our understanding of the mechanisms regulating TNF responses in the liver. Although JNK activation facilitates TNF-induced apoptosis in NF-κB deficient liver cells, the results show that even excessive JNK activity is not sufficient to sensitize hepatocytes with intact NF-κB signaling to TNF toxicity. However, when p38α deficiency is combined with the ablation of IKK2, lack of negative JNK regulation synergizes with the partial impairment of NF-κB activity, to sensitize the liver to endotoxin-induced failure (Fig. 10).

4.2 The role of liver p38α in chronic low grade inflammatory states. A model of high fat diet induced obesity and insulin resistance.

Obesity, insulin resistance and type 2 diabetes are closely associated with chronic inflammation that involves abnormal cytokine production, the increase of acute-phase reactants and activation of a network of inflammatory signaling pathways (Wellen and Hotamisligil 2005). The combinatorial and additive action of different signaling pathways in metabolic homeostasis is a crucial but poorly addressed subject. In order to understand the contribution of inflammatory pathways that are downstream of TNF receptor signaling it is important to investigate their contribution in peripheral organs like the liver, muscle and adipose tissue. The liver is an important contributor to the normal regulation of glucose homeostasis. The ability of insulin to inhibit hepatic gluconeogenesis is an important aspect of insulin-stimulated clearance of blood glucose. The liver is therefore a potential site of metabolic regulation that influences insulin resistance. In the liver, pathways like JNK and NF-κB signaling were investigated intensively in chronic subacute inflammatory states induced by obesity showing that both molecules have a negative effect on insulin signaling (Hirosumi, Tuncman et al. 2002; Arkan, Hevener et al. 2005; Cai, Yuan et al. 2005). The p38 signaling pathway has not been investigated for its contribution to low grade inflammatory states in the liver induced by obesity. Therefore, in the second part of this thesis the effect of a liver specific deletion of p38α in a high fat diet induced model of obesity and insulin resistance was investigated.

4.2.1 $p38\alpha^{LPC\text{-}KO}$ are more insulin resistant than control mice

$p38\alpha^{LPC\text{-}KO}$ that were placed on HFD for 16 weeks, did not gain more weight on a HFD than control mice. Therefore, we concluded that the liver parenchymal knockout of p38α did not alter energy homeostasis (Fig. 11). Moreover, the liver parenchymal knockout of p38α did not cause any differences in blood glucose levels indicating that the metabolic phenotype of these mice is rather mild (Fig. 12 A). Similar to blood glucose levels, serum insulin levels were higher in HFD treated animals compared to animals that received chow diet, indicating a higher degree of insulin resistance. Interestingly, after chow diet there was a trend towards higher levels in $p38\alpha^{LPC\text{-}KO}$ mice, suggesting that these mice may need to secrete more insulin to maintain normal glucose levels in order to compensate for a potential metabolic dysfunction in the liver. In an insulin resistant state, after HFD, where insulin levels are generally higher, this trend was not observed (Fig. 12 A). Furthermore, no differences in

serum triacylglyeride and cholesterol levels were observed, indicating that the secretion of lipids was not altered in mice deficient for p38α in liver parenchymal cells (Fig. 12 C, D).
In the glucose tolerance tests obese p38α^{LPC-KO} mice failed to clear the injected glucose as efficient as control mice (Fig. 13 A). The insulin tolerance test showed a significantly impaired response to insulin in obese p38α^{LPC-KO} mice (Fig. 13 B). Thus, obese p38α^{LPC-KO} mice are more insulin resistant than control mice. Increased amounts of hepatic gluconeogenesis are likely to be responsible for this phenotype. Unfortunately, clamp studies to determine the hepatic glucose production and disposal of glucose in the peripheral tissues could not be realized in this study. *In vivo* insulin injections, investigating the response of the different peripheral organs to insulin, indicated a local insulin resistance in the liver caused by the deletion of p38α, while the muscle was not affected (Fig. 14 A, B). Moreover, p38α deficient primary hepatocytes stimulated with insulin showed clearly impaired insulin mediated signaling (Fig. 15). Thus, the deletion of p38α in hepatocytes leads to an impairment of insulin signaling and is responsible for the increased insulin resistance in obese p38α^{LPC-KO} mice.

4.2.2 Lipids and the expression of inflammatory cytokines do not contribute to the development of insulin resistance

Local insulin resistance can be caused by several mechanisms. One of them is the accumulation of toxic lipid intermediates like diacylglycerols (DAGs) but also storage lipids like triacylglycerols (Samuel, Liu et al. 2004; Neschen, Morino et al. 2005; Zhang, Liu et al. 2007). DAGs accumulate in skeletal muscle and liver when the rate of fatty acid delivery to these tissues exceeds the rate of intracellular fat oxidation and/or conversion to neutral lipids. Increased intracellular DAG levels lead to activation of PKC-θ and PKC-ε in skeletal muscle and liver, respectively, which in turn, decreases insulin-stimulated IRS-1/IRS-2 tyrosine phosphorylation, PI3K activation and downstream insulin signaling. To test if a dysregulation in liver lipids contributes to the elevated insulin resistance, liver lipid quantity and quality of p38α^{LPC-KO} mice was compared to control mice. Histology and thin layer chromatography of livers did not show any differences in liver lipid quality and quantity. Thus, impaired insulin signaling in the livers of p38α^{LPC-KO} mice does not seem to be caused by a difference in lipid content, a factor known to impair insulin signaling (Fig. 16 A, B, C, D).

Furthermore, the expression of inflammatory cytokines in the livers of p38α^{LPC-KO} mice was assesed. Elevated expression of inflammatory cytokines by resident and infiltrating immune cells or by hepatocytes themselves can potentially lead to an increase of insulin resistance (Hotamisligil 2006). Hepatocyte specific deletion of IKK2 was shown to protect mice from diet induced insulin resistance due to a decreased expression of inflammatory cytokines in the liver (Arkan, Hevener et al. 2005). Therefore, an elevated expression of inflammatory

cytokines in p38α deficient livers could potentially cause a local insulin resistance through the activation of inflammatory signaling pathways. Quantitative analysis of cytokine expression in the livers of p38α^{LPC-KO} mice did not reveal any significant differences in TNF, IL-1-β or IL-6 expression (Fig. 17). TNF, IL-1β or IL-6 are heavily implicated in causing insulin resistance through impairment of insulin signaling (Hotamisligil 1999; Larsen, Faulenbach et al. 2007; Sabio, Das et al. 2008). Conclusively, we did not find any indication that an altered liver expression of TNF, IL-1-β or IL-6 might be the cause for decreased insulin signaling in p38α deficient livers.

4.2.3 Increased activation of JNK in the livers of p38α^{LPC-KO} mice

During the biochemical characterization of p38α deficient livers taken from obese mice a significant hyperactivation of JNK was detected (Fig. 19 A). In agreement with the LPS/TNF induced model of acute liver inflammation, liver p38α also negatively regulates JNK in chronic low grade inflammatory states. It is also noteworthy that this hyperactivation of JNK was also observed in resting primary hepatocytes. Therefore, p38α$^{-/-}$ cells have elevated intrinsic JNK activity regardless of the stimulus (Fig. 19 B).

JNK has been implicated as a negative regulator of insulin signaling in various studies. JNK1$^{-/-}$ mice were shown to be protected from diet induced obesity and insulin resistance (Hirosumi, Tuncman et al. 2002). Also, an interaction between JNK1 and the insulin receptor substrate was shown, leading to its negative regulatory phosphorylation (Solinas, Naugler et al. 2006) Nevertheless, new data generated with conditional JNK1 knockout mice puts previous publications generated with JNK1$^{-/-}$ mice into a different light. The protection of JNK1$^{-/-}$ mice against diet induced obesity seems to be caused by a reduced somatic growth in the presence of reduced circulating growth hormone (GH) and insulin-like growth factor 1 (IGF1) concentrations, as well as increased thyroid axis activity (Belgardt, Mauer et al.). There is controversial data about the involvement of JNK1 in the hematopoetic compartment (Solinas, Vilcu et al. 2007; Sabio, Das et al. 2008; Vallerie, Furuhashi et al. 2008). In the adipose tissue JNK1 regulates the expression of IL-6 that seems to impair insulin signaling in the liver but not in adipose tissue or muscle (Sabio, Das et al. 2008). In the muscle, JNK1 seems to play a role in the suppression of insulin signaling (Sabio, Kennedy et al.). The liver specific knockout of JNK1 revealed a positive role for JNK1 in insulin signaling, (Das, Sabio et al. 2009) or had no phenotype (Wunderlich *et al*, unpublished data).

4.2.4 Inhibition of JNK can only partially restore insulin signaling in p38α deficient hepatocytes

Due to the hyperactivation of JNK in p38α deficient livers, primary hepatocytes were treated with a JNK inhibitor, before insulin stimulation, in an attempt to rescue the impaired signaling. Despite clearly reduced JNK activation as seen from the reduced c-Jun phosphorylation after inhibitor application, insulin signaling could only be restored to a very mild degree (Fig. 20). This supports studies claiming that JNK signaling in hepatocytes may not have a strong effect on insulin signaling (Das, Sabio et al. 2009) (Wunderlich *et al*, unpublished data)..

4.2.5 Increased phosphorylation of Ser307 in p38α deficient hepatocytes

The negative regulatory phosphorylation of the IRS1 has been discussed as a major mechanism by which inflammatory kinases inhibit insulin signaling. Accumulating evidence shows that JNK and other kinases can promote increased IRS1 phosphorylation at Ser 307 leading to an impairment of insulin signaling (Aguirre, Uchida et al. 2000; Aguirre, Werner et al. 2002; Hirosumi, Tuncman et al. 2002; Lee, Giraud et al. 2003). Recently, controversy has been raised by several *in vivo* studies investigating the role of serine residues on IRS1 in the transduction of the insulin signal. On the one hand germ-line mutational analysis in mice demonstrates that Ser-307 on IRS1 is not essential for insulin resistance, *in vivo* (Copps, Hancer et al.). On the other hand the mutation of three serine residues of the IRS1 in the muscle protected mice from the development of diet induced obesity and insulin resistance (Morino, Neschen et al. 2008).

Independent from this controversy, the phosphorylation status of IRS1 at serine 307 was examined. Indeed, as shown in Fig. 21 p38α deficient hepatocytes show increased phosphorylation of IRS1 at serine 307. A JNK inhibitor was applied to decrease the phosphorylation of IRS1 at serine 307, in p38α deficient hepatocytes. Similar to insulin receptor signaling, the application of the JNK inhibitor failed to reduce the phosphorylation of IRS1 at serine 307, suggesting that other kinases might be involved in this hyperphosphorylation. It also has to be considered that the insulin receptor substate 1 and 2 have a high degree of redundancy (Kubota, Kubota et al. 2008). Thus, the status of the IRS2 protein, which was not addressed in this study, remains to be investigated. Furthermore, IRS independent effects that impair insulin mediated signaling cannot be ruled out and have to be investigated in further detail (Hoehn, Hohnen-Behrens et al. 2008).

Finally, the activation of the upstream kinases MKK4, ASK1 and TAK1 was tested. All these upstream kinases seem to be hyperactivated in p38α deficient hepatocytes (Fig. 21). This suggests that a whole set of kinases is hyperactivated in p38α deficient hepatocytes, which could orchestrate the increased phosphorylation of IRS1 at serine 307. In this case, it might not be JNK alone leading to the impairment of insulin signaling but a whole set of kinases, including TAK1, which is very proximal to the TNF receptor. TAK1, MKK4 and ASK1 have not been implicated in the development of insulin resistance thus far. Future studies are required to show if indeed one of these kinases can dampen insulin mediated signals in p38α deficient hepatocytes.

In conclusion, deletion of the p38α MAPK in liver parenchymal cells results in decreased glucose tolerance and increased insulin resistance in obese mice. p38α deficient livers and isolated primary hepatocytes show decreased insulin signaling. Liver lipids remain unaffected, ruling out a possible accumulation of lipid intermediates that could negatively regulate insulin signaling. Moreover, an elevated expression of the inflammatory cytokines TNF, IL-1β or IL-6 in the liver, was not detected. Increased activation of JNK and its upstream kinases MKK4, TAK1 and ASK1 is likely to be responsible for causing increased insulin resistance in p38α-deficient hepatocytes, through an unknown mechanism. Therefore, in obese mice, liver p38α may have an important function regulating glucose homeostasis, by controlling the activity of kinases that might be responsible for the negative regulation of insulin signaling. These results also strongly argue against the administration of JNK inhibitors to treat chronic inflammatory states in obese individuals (Kumar, Boehm et al. 2003).

5. Perspectives

In this study liver p38α was shown to have very important functions in chronic and acute inflammatory conditions. In acute inflammatory states, induced by LPS injection p38α, collaborates with IKK2 to protect the liver from LPS/TNF-induced failure by controlling JNK activation. In chronic inflammatory states, caused by diet induced obesity p38α ensures a better glucose homeostasis by controlling kinases in the JNK signaling pathway.

In both studies, the molecular basis for the hyperactivation of these kinases could not be identified so far. Future studies are required to show how p38α maintains the balance between the kinases, keeping the liver in homeostasis. Here, evidence points to a defect which seems to be very proximal to the TNF receptor itself since MAP3Ks like ASK1 and especially TAK1 are hyperactivated. This could be caused by defective scaffolding or the lack of a negative regulatory process at the receptor complex itself, like a constitutive degradation of TRAF molecules allowing a constant signal to evolve. Both possibilities can be explored by biochemical methods like immunoblotting or co-immunoprecipitation. Also the involvement of phosphatases cannot be ruled out at this stage. To this end, a reliable phosphatase assay should be established to test the activity of candidate phosphatases.

The LPS model of acute liver inflammation leaves the question whether other anti-apoptotic molecules are involved in preventing LPS/TNF induced apoptosis. Since the partial inhibition of NF-κB sensitizes livers to LPS/TNF induced toxicity, anti apoptotic NF-κB target genes including members of the IAP family are likely to play a role.

In the diet induced obesity model, clamp studies should be performed to identify increased hepatic glucose production as the cause for decreased glucose tolerance and insulin sensitivity in the liver.

Furthermore, the ability of the hyperactivated kinases TAK1, ASK1 and MKK4 to impair insulin mediated signaling has to be determined in animal studies. Also, since the defect in signaling is so proximal to the TNF receptor the question for the liver specific role of the TNFR1 in diet induced obesity and insulin resistance rises and should be addressed in a separate conditional mouse model.

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