Role of Bid in Liver injury following BDL and CCl4-induced liver damage

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Genehmigte Dissertation

Von

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The following study has been carried out under the supervision of Dr. med. Arndt Vogel, Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany between October 2006 and October 2009.

Declaration: Here with I declare that the study has been done by my own under the guidance of Dr. med. Arndt Vogel and all the information provided is novel and true and has not been submitted to any other institute or University to obtain any other degree.

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Contents

Abstract:

Death receptor-mediated hepatocyte apoptosis has been implicated in a variety of acute and chronic liver diseases. The BH3-interacting domain death agonist Bid is a critical mediator for apoptosis induced by activation of Fas and TNF-R1 death receptors in hepatocytes. The aim of this study was therefore to elucidate the role of Bid in cholestatic and toxic liver injury.

Accumulation of bile acids during obstructive cholestasis causes liver injury and fibrosis, which is at least partly mediated by the death receptors Trail, Tnf-α and Fas. To analyse the role of Bid in cholestatic liver injury, overall survival and various aspects of liver injury were analyzed in WT and *Bid*$^{-/-}$ mice following bile duct ligation, a commonly used model to study obstructive cholestasis in mice. Loss of Bid did not affect number of bile infarcts, serum AST values and animal survival. Importantly, *Bid*$^{-/-}$ mice displayed the same pattern of TUNEL positive hepatocytes as WT controls. Processing of procaspase-3 and -9 and caspase-3 enzyme activities however were not detectable in either group. In contrast to Fas-receptor deficient lpr mice, hepatic fibrosis and the inflammatory response was not affected by loss of Bid. Together, these data suggest that Bid does not play a role in cholestatic liver injury.

To analyse the role of Bid in toxic liver injury, WT and *Bid*$^{-/-}$ mice were challenged with *CCl4.* Previously, it has been proposed that CCl4 does not only causes necrosis, but also induces apoptosis in mouse livers, which might sustain inflammation and liver injury. Here we show that loss of Bid ameliorates CCl4 induced liver injury even though CCl4 did not induce significant hepatocyte apoptosis. Reduced liver injury after a single CCl4 injection correlated with an attenuated inflammatory response, which might contribute to the observed phenotype. Surprisingly, loss of Bid caused more severe liver injury in mice treated for 6 weeks with CCl4 suggesting that Bid is required for the adaptation of hepatocytes against chronic liver injury induced by CCl4. Additional studies will be necessary to further dissect the role of Bid in toxic CCl4-induced liver injury. Our data however indicate that Bid plays a role in the liver beyond its role in mediating hepatocyte apoptosis.

Key words: Bid, BDL and CCl4

Abstrakt:

Todesrezeptor-vermittelte Apoptose von Hepatozyten spielt eine wichtige Rolle in einer Reihe von akuten und chronischen Lebererkrankungen. Bid (BH3-interacting domain death agonist) ist ein kritischer Vermittler für Apoptose, die durch Aktivierung von Fas und TNF-R1 Todesrezeptoren in Hepatozyten induziert wird. Ziel dieser Studie war es deshalb die Rolle von Bid in cholestatischen und toxischen Leberschädigungen zu ermitteln.

Die Akkumulation von Gallensäuren während obstruktiver Cholestase verursacht eine Leberschädigung und -fibrose. Um die Rolle von Bid während einer cholestatischen Leberschädigung zu analysieren, wurden das Überleben und verschiedene Aspekte der Leberschädigung in WT und *Bid*$^{-/-}$ Mäusen nach Gallengangligatur, ein etabliertes Modell zur Untersuchung der obstruktiven Cholestase in Mäusen, analysiert. Der Verlust von Bid beeinflusste weder die Anzahl von Galleninfarkten und die Höhe der AST Serumwerte noch das Überleben der Tiere. Interessanterweise fanden sich zudem in Lebern der *Bid*$^{-/-}$ Mäuse die gleiche Anzahl und Verteilung von TUNEL positiven Hepatozyten im Vergleich zu den WT Kontrollen. Eine Prozessierung von Procaspase-3 und -9 und Caspase-3 Enzymaktivitäten war jedoch in keiner der Gruppen detektierbar. Anders als in Fas-Rezeptor defizienten lpr Mäusen wurden durch den Verlust von Bid weder die hepatische Fibrose noch die inflammatorische Antwort beeinflusst. Zusammenfassend lässt sich vermuten, dass Bid keine Rolle in der cholestatischen Leberschädigung spielt.

Um die Rolle von Bid in der toxischen Leberschädigung zu analysieren, wurden WT und Bid-/- Mäuse mit CCl4 behandelt. Früheren Arbeiten lassen vermuten, dass CCl4 nicht nur Leberzellnekrosen auslöst, sondern auch Apoptose von Introduction Hepatozyten induziert, wodurch möglicherweise die Entzündungsreaktion und damit die Leberschädigung verstärkt wird. Wir konnten in dieser Arbeit zeigen , dass der Verlust von Bid zu einer Verbesserung des durch CCl4 induzierten Leberschadens führt, obwohl CCl4 keine signifikante Apoptose in Hepatozyten auslöste. Die reduzierte Leberschädigung nach einfacher CCl4-Injektion korrelierte mit einer verzögerten Immunantwort, welche zu dem beobachteten Phänotyp beitragen könnte. Überraschenderweise verursachte der Verlust von Bid einen stärkere Leberschädigung

in Mäusen, die über sechs Wochen mit CCl4 behandelt wurden, was die Vermutung nahelegt, dass Bid für die Adaption von Hepatozyten gegenüber CCl4-induzierter chronischer Leberschädigung beiträgt. Zusätzliche Studie werden notwendig sein, um die exakte Rolle von Bid im Rahmen der CCl4-induzierten Leberschädigung zu klären. Insgesamt lassen unsere Ergebnisse aber vermuten, dass Bid in Hepatozyten nicht nur Apoptose induziert, sondern noch weitere biologische Funktionen ausübt.
Schlagworter: Bid, BDL and CCl4

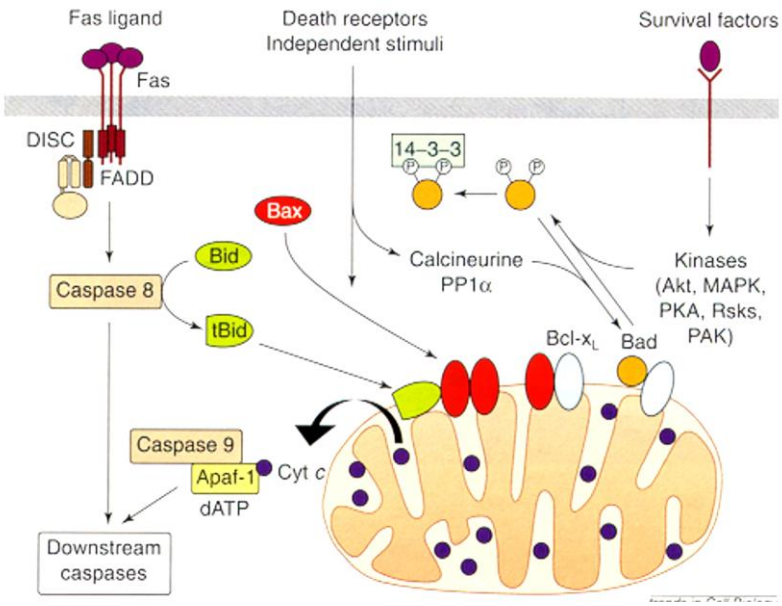
1. Introduction:

1.1 Apoptosis:

Apoptosis, programmed cell death (PCD), is a morphologically and biochemically distinct form of cell death. Apoptosis can be characterized by membrane blebbing, chromatin condensation and nuclear fragmentation. During apoptosis the intracellular organelles remain intact in contrast to necrosis. Apoptosis may occur by two pathways, the death receptor or extrinsic pathway and the mitochondrial or intrinsic pathway.

1.1.1 Death receptor pathway or extrinsic pathway:

The most extensively characterized death receptors are CD95 (Fas or Apo1) and CD120a (TNF-R1) (Trauth et al., 1989); (Ito et al., 1991); (Ashkenazi and Dixit, 1998),. Triggering of the CD95/Fas molecule either by agonistic antibodies or by the natural ligand FasL induces trimerization of the receptor, and the trimerized cytoplasmic region then transduces the signal into the cell by recruiting a molecule called FADD (Fas-associating protein with death domain) or MORT1 (mediator of receptor- induced toxicity), which binds to CD95 via interaction of the death domain at its COOH terminus (Chinnaiyan et al., 1996). The NH_2 terminal region of FADD is responsible for downstream signal transduction by recruitment of a protein called FLICE (FADD Like Interleukin-1*b* Converting Enzyme) or MACH (MORT1-Associated CED-3 Homologue), designated as caspase-8 (Boldin et al., 1996).



The NH_2 terminus of caspase-8 binds to FADD/MORT1, (while its COOH-terminal region is

related to the caspase-3 (CPP32) subfamily) and allowing procaspase- 8 and -10 activation by auto processing (Boldin et al., 1996); (Muzio et al., 1996); (Kischkel et al., 2001); (Wang et al., 2001); (Sprick et al., 2002); (Aouad et al., 2004). In so called type I cells active caspase-8 can directly cleave effector caspases, such as caspases 3 and 7, leading to apoptosis.

1.1.2 Mitochondrial or intrinsic pathway:

Mitochondria can release several death-promoting factors in response to extrinsic and intrinsic death signals. A number of death-promoting factors have been identified in this pathway, including cytochrome C (Liu et al., 1996), AIF, Smac (also known as DIABLO), endonuclease G. Whereas AIF and endonuclease G seem to be able to directly cause nuclear and DNA damage, cytochrome C together with Apaf-1 to activate another initiator caspase, caspase-9, which then activates the effector caspases.

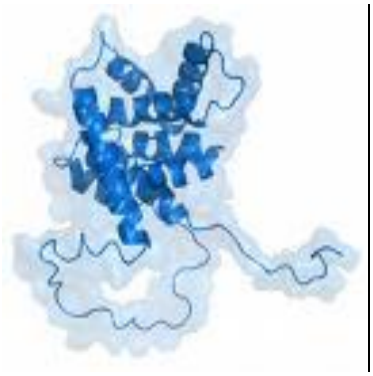
1.2 The Bcl-2 family proteins as important apoptosis regulators:

Bcl-2 family proteins regulate apoptosis via the mitochondrial pathway. Bcl-2 family of proteins share structural homology in BH1 to BH4, which are structural basis for their function and also to interact in between family members to execute their function. Bcl-2 family of proteins controls MOMP (Mitochondrial Outer Membrane Potential) and can be either pro-apoptotic (Bid, Bad, Bax, Bak etc) or anti-apoptotic (Bcl-2 proper, Bcl-xL, Bcl-w etc). At first, pro-apoptotic proteins like Bid, Bad etc. receive the signal from cell surface via the receptors and amplify the death signal via mitochondrial pathway by releasing cytochrome C. While anti-apoptotic proteins, like Bcl-2 and Bcl-xL suppress cytochrome C release (Yang et al., 1997). Over expression of anti-apoptotic Bcl-2 family members, such as Bcl-2 or Bcl-xL (Strasser et al., 1991) or the combined loss of the pro-apoptotic Bcl-2 family members Bax and Bak renders cells resistant to many apoptotic stimuli, including cytokine deprivation or over expression of BH3-only proteins (Wei et al., 2001).

1.3 Bid Protein and its role in liver:

BID (**B**cl-2 **I**nteracting **D**omain) is a 22 kDa pro-apoptotic protein and is a member of the Bcl-2 homology domain (BH)-3- family of pro-apoptotic proteins that initiates apoptotic cell death (Puthalakath and Strasser, 2002). The BH3 domain of Bid is essential for its death activity.

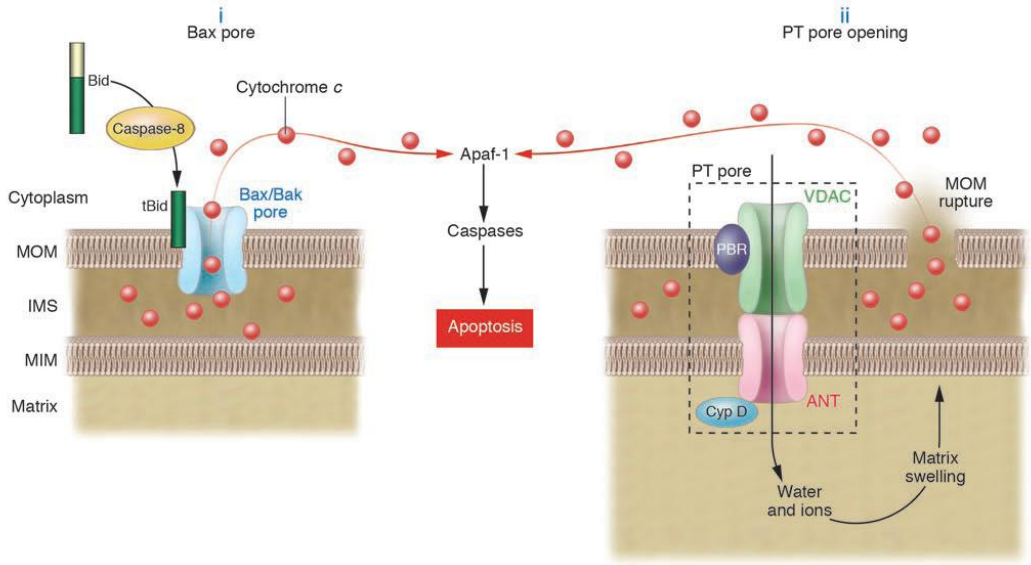
Bid protein



However, Bid, unlike most other members of the Bcl-2 family, lacks a COOH-terminal membrane-anchoring segment and is largely present in cytosol.

1.4 Molecular mechanisms of Bid in mitochondrial pathway:

In type II cells activation of caspase-8 at the DISC complex is insufficient to trigger cell death and a mitochondrial amplification loop via the mitochondria is required, which is mediated by Bid. Cleavage of Bid by capsase-8 activates Bax, which normally resides in the cytosol, then translocates to mitochondria when triggered by certain stimuli (Eskes et al., 1998), (Wolter et al., 1997). Here tBid activates Bax/ Bak. Bax or Bak forms a pore in the MOM (Mitochondrial Outer Membrane). Bid induces Bax oligomerization and integration in the outer mitochondrial membrane (Eskes et al., 2000), that allows an influx of water and ions into the matrix, causing matrix swelling; this leads to rupture of the mitochondrial outer membrane potential often called as MOMP, releasing inner mitochondrial space (IMS) protein cytochrome C (Wei et al., 2000). Cytochrome C stimulates the assembly of a multi protein complex known as the Apaf-1 apoptosome. Caspase-9 is recruited to the apoptosome and activated (Adrain and Martin, 2001), this complex further activates caspase-3 by cleaving. Cleaved caspase-3 and or caspase-7 cleave nuclear lamins, causing the nucleus to break down and lose its normal structure and thus apoptosis. It transduces a downstream signal cascade via caspases resulting in apoptosis. There is also possibility rarely that even though if the caspases are inhibited there is disruption of MOMP and eventually cell death.



Review by Lisa Bouchier-Hayes in JCI 2005; Molecular mechanisms of MOMP.
The proposed models of MOMP leading to cytochrome C release are represented

1.5 Role of Bid in Fas induced apoptosis:

To study the loss of Bid protein in Fas mediated apoptosis, Xiao-Ming Yin (Yin et al., 1999) developed *Bid*$^{-/-}$ mice. *Bid*$^{-/-}$ mice have no developmental abnormalities, and have normal spleens, thymuses, brains, hearts, livers, lungs, kidneys and testes. To determine whether Bid is required *in vivo* for cytochrome C release, mitochondrial dysfunction or death of cells, they injected mice intravenously with anti-Fas antibody (Jo-2; 0.25 mg g-1). Most *wild type* mice died within 4 h of acute liver failure associated with massive hepatic apoptosis and necrosis. A single intravenous injection of anti-Fas antibody (Jo-2) (Ogasawara et al., 1993) induced cleavage of Bid in the liver and caused its translocation to mitochondria (Gross et al., 1999). The cleaved fragment of Bid was first detected in cytosol 1 h after injection, but was found completely in mitochondria 3 h after injection. In contrast to *wild type*, most *Bid*$^{-/-}$ mice survived anti-Fas antibody injection, about half of the mice had no detectable liver injury by gross or microscopic examination, and there was no evidence of apoptosis from nuclear morphology or TUNEL staining. In this study they demonstrated that Bid is an indispensable substrate for selected cells and death agonists, as shown by the hepatocyte apoptosis induced by Fas *in vivo*.

1.6 Role of Bid in FasL/Mega Fas induced apoptosis:

FasL is synthesized as a type II–membrane protein, which undergoes metalloproteinase mediated proteolytic cleavage resulting in the release of soluble trimeric ligand (Tanaka et al., 1996). Interestingly, some *in vitro* studies argued against the two-pathway concept because the differences were not found when cells were stimulated with FasL rather than with an agonistic antibody and questioned that antibodies accurately reflect Fas-signaling induced by the physiological ligand and are therefore of clinical relevance (Huang et al., 1999). When freshly isolated mouse hepatocytes from *Wild type* and *Bid*$^{-/-}$ mice cultured on to the collagen or matrigel surface and treated with FasL, same level of cell injury is seen in both the genotypes. It means FasL-induced caspase-3 activation and apoptosis of collagen plated hepatocytes does not depend on Bid, where Fas/FasL signaling switched from type II to type I pathway after the treatment with FasL. Surprisingly when cells are in suspension there was no switching of the pathway (Walter et al. 2008) is seen with FasL. So, here hepatocytes when plated on collagen surface they are switching from Bid dependent to Bid independent pathway. It seems extracellular matrix components can cause switching of the pathway. In one recent study it was shown that the strength of the Fas signal determines whether hepatocytes act as type I or II cells in murine livers. Hepatocytes challenged with the anti-Fas antibody Jo-2 require Bid to undergo apoptosis as previously reported. Remarkably however, loss of Bid did not completely inhibit apoptosis induced by Mega FasL (Schungel et al., 2009). Mega FasL is a hexameric form of sFasL.

1.7 Role of Bid in Tnf-R mediated apoptosis:

It has been showed that in both experimental conditions TNF-alpha (Tnf-a) is important for the hepatocyte killing and animal death (Pfeffer et al., 1993). In order to study the mechanism underlying Tnf-R mediated pathway, method is to inject the mice are injected with low doses of LPS (bacterial lipoploysaccharide) together with the liver-specific transcriptional inhibitor D-(+)-galactosamine (GalN). Like in Fas mediated apoptosis, Tnf-R (Tnf-R also has an intracellular death domain) activation triggers apoptosis by binding the adaptor proteins TRADD and FADD, which facilitate binding and activation of caspase-8 (Ashkenazi and Dixit, 1998). The rest of the pathway is similar to Fas mediated apoptosis. In contrast to FasL induced apoptosis, loss of Bid does not protect hepatocytes from LPS and GalN induced apoptosis. Loss of Bid in Fas-L induced hepatocyte killing after combined treatment of LPS and GalN resulted only in a minor reduction of liver damage. However, combined loss of Bid and another BH3-only protein, Bim, activated by c-Jun N-terminal

kinase (JNK), protected mice from LPS+ GalN-induced hepatitis/ liver damage. TRADD also recruits other molecules, such as TRAF2, which actually activates a protective pathway through the transcription factor nuclear factor kB (Nf-kB). In many types of cells, TNF induces cell death only in the presence of transcriptional inhibitors.

1.8 Role of apoptosis/Bid in liver disease:

Hepatocyte apoptosis, mainly induced by death receptor ligands such as TNF-a and FasL, is implicated in several experimental and human liver diseases including viral hepatitis, alcoholic liver disease, acute liver failure, ischemia–reperfusion (I-R) injury, graft rejection, diseases of the bile ducts, and hepatocellular carcinoma (HCC). Therefore, identification of pro- and anti-apoptotic pathways in death receptor-mediated hepatocyte apoptosis would contribute to understanding of the pathophysiological role of apoptosis in liver diseases.

1.8.1 Cholestasis/ BDL:

Cholestasis refers to the condition where no bile flows from liver to intestine and retained within the liver, causing severe damage. In experimental condition cholestasis can be mimicked by bile duct ligation where common bile duct is ligated in order to stop the bile flow. This model induces biliary type fibrosis similar to that which can be seen in some human liver diseases. BDL stimulates the proliferation of biliary epithelial cells and oval cells (which are hepatocytes progenitors), resulting in proliferating bile ductules with an accompanying portal inflammation and fibrosis (Tsukamoto et al., 1990) (Iredale et al., 1996). Therapeutic options for the treatment of cholestatic liver injury are currently limited, and a better understanding of the mechanisms leading to cellular injury may help to develop new therapeutic strategies.

Multiple studies have shown that bile acids induce Fas-dependent hepatocyte apoptosis *in vitro* providing a cellular mechanism for bile acid induced liver injury (Faubion et al., 1999); (Miyoshi et al., 1999); (Higuchi et al., 2001); (Reinehr et al., 2003); (Roberts et al., 1997). Additionally, it has been shown that death receptor mediated apoptosis does not only cause hepatocyte death *in vivo* but also contributes to stellate cell activation and liver fibrogenesis in cholestatic liver injury (Canbay et al., 2002); (Miyoshi et al., 1999); (Schoemaker et al., 2003); (Sodeman et al., 2000). Upon ligand binding, activated death receptors engage the Fas associated death domain adaptor protein (FADD). FADD in turn recruits caspase-8 via a homophilic death effector domain (DED) interaction forming the death-inducing signalling complex (DISC) leading to activation of caspase-8. In type-II cells

such as hepatocytes, activation of caspase-8 at the DISC alone is insufficient to efficiently trigger cell death and an amplification loop via the mitochondria is required (Scaffidi et al., 1998). Previous studies have shown that this link between the death receptor and the mitochondria is dependent on cleavage and translocation to the mitochondria of the pro-apoptotic Bcl-2 family member Bid (Li et al., 1998; Luo et al., 1998). During the apoptotic process, Bid activates Bax or Bak to initiate mitochondrial dysfunction, release of cytochrome C and activation of effector caspases such as caspase-3 (Acehan et al., 2002). The role of death receptors in cholestatic liver injury is supported by studies showing that mice deficient in Fas-receptor develop less liver injury following bile duct ligation (BDL) (Miyoshi et al., 1999); (Gujral et al., 2004), a widely used rodent model of cholestasis. Interestingly, reduced liver injury in BDL lpr mice correlated with a less severe inflammatory response (Gujral et al., 2004). Likewise, liver histology, number of bile infarcts, serum AST values, hepatic fibrosis, and animal survival were significantly improved in BDL *TRAIL*$^{-/-}$ mice as compared to *wild type* controls (Kahraman et al., 2008; Takeda et al., 2008). Finally, a very recent study revealed that TNF-α also promotes hepatotoxicity in the BDL model and that *TNF-*$\alpha^{-/-}$ mice develops significantly less liver fibrosis than littermate controls (Gabele et al., 2009). Together, these studies suggest that all three members of the death receptor family contribute to bile acid induced liver injury and fibrosis. However, it is still unclear which down-stream effectors of the death receptors mediate the deleterious effects in murine livers following BDL. Previously, it has been shown that Bid antisense molecules reduce the acute liver injury following obstructive cholestasis suggesting that Bid might be an important down-stream target of death receptors in obstructive cholestasis (Higuchi et al., 2001).

1.8.2 CCl4 model:

Administration of carbon tetrachloride (CCl4) to rodents is a widely used model to study chemical toxin induced hepatic injury. CCl4 causes hepatocytes injury that is characterized by centrilobular necrosis which is followed by hepatic fibrosis. Carbon tetrachloride causes liver damage by several mechanisms, including disruption of membrane permeability, generation of free radicals and release of large amounts of cytokines like Tumor necrosis factor alpha (TNF-α) (Berger et al., 1986), (Czaja et al., 1989). Moreover, it has been shown that a single dose of CCl4 induces not only liver necrosis, but also hepatocyte apoptosis (Shi et al., 1998). Intoxication with CCl_4 results in hepatocyte damage, necrosis, inflammation, and fibrosis, which spreads to link the vascular structures and over 8–12 weeks results in the development of cirrhosis (Constandinou et al., 2005)(Constandinou, C., 2005).

Hepatic injury due to CCl4 is thought to involve two phases (Shi et al., 1998)(Shi J et al. 1998). First, CCl4 is metabolized by cytochrome P450 in hepatocytes, giving rise to highly reactive trichloromethyl free radicals. In one study, the involvement of CYP2E1 in CCl4-induced hepatotoxicity was investigated *in vivo* using *cyp2e1*-/- mice which lack CYP2E1 expression (Lee et al., 1996). Mice which lack CYP2E1 expression were resistant to liver damage after exposure to CCl4 indicating that CYP2E1 is a major factor involved in CCl4-induced hepatotoxicity *in vivo* (Wong et al., 1998). Serveral cytokines and chemokines are activated during the CCl-induced inflammatory response ((Laskin and Pendino, 1995). These cytikine secreted by various non-parenchymal liver cells such as Kupffer cells (KCs), hepatic stellate cells (HSCs) and sinusoidal endothelial cells (SECs) contribute not only to liver injury but also to liver fibrogenesis. Furthermore, activation of Kupffer cells by engulfment of apoptotic bodies leads to the release of pro-inflammatory cytokines, nuclear factor-kappa B (NF-κB) activation ((Li et al., 2005), expression of pro-apoptotic ligands and further hepatocytes apoptosis which may contribute to the continuous inflammatory status in the liver. Several functions have been attributed to cytokines, including activation of HSCs, modulating expression and deposition of matrix proteins and regulating the regeneration of hepatocytes. HSCs become activated and proliferate intensely, senesce, and are eventually cleared to protect the liver from an excessive fibrogenic response to acute injury (Krizhanovsky et al., 2008). Therefore, resolution of liver fibrosis could be associated with the down regulation of inflammatory responses mediated by cytokines (Friedman, 1999); (Koziel, 1999). Among the cytokines, transforming growth factor (Tgf ß1) is associated to the activation of HSC and the following production of ECM (Bissell, 2001). Apart from Tgf ß1, TNF-a and IL-6 may play key roles during CCl4-induced hepatic fibrogenesis (Zhang et al., 2004). In hepatocytes, after triggering with CCl4, oxidative damage is one of the essential mechanisms of hepatotoxicity induced by CCl4. Moreover, mitochondria-initiated apoptosis triggered by ROS plays an important role in this hepatotoxicity in rat primary hepatocytes (Cai et al., 2005).

1.9 Summary and focus of the current study:

The overall objective of the study was to examine the role of Bid, one of the best-known down-stream mediators of death receptors in type II cells, in cholestatic and toxic liver injury (Shi et al., 1998). We were specifically interested to determine to which extent Bid-mediated mitochondrial pathway affects overall survival, liver injury and hepatic fibrogenesis in *wild type* and *Bid*$^{-/-}$ mice in both liver injury models.

2. Materials and methods:

2.1. Mice:

C57Bl/6 mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). *Bid*$^{-/-}$ mice were provided by S. Korsmeyer. Floxed c-Jun mice were provided by E. Wagner, *c-Jun*$^{f/f}$ mice were crossed with transgenic mice expressing *Mx-Cre*. *Mx-Cre c-Jun*$^{f/f}$ mice were injected twice with poly (I/C) 10 days before the experiment (15 mg/kg i.p.; Amersham Biosciences, Piscataway, NJ) to obtain mice lacking c-Jun. Fas receptor deficient *lpr* mice and age-matched C57BL/6J *wild type* mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). Animal experiments were approved by the district government of Lower Saxony, Germany. Experiments were performed with 7-8 weeks old mice weighing around 25g. Mice were housed with a 12:12h light-dark cycle and permitted consumption of water and a standard mouse diet ad libitum.

Bile duct ligation was performed as previously described. In brief, mice were anesthetized and the peritoneal cavity was opened. The common bile duct was double ligated and cut between the ligatures. Control mice underwent sham operation with exposure, but without ligation of the common bile duct. The mice were sacrificed after 3, 7 and 14 days. At the time of sacrifice, a small fragment of each liver was fixed in buffered formalin. The remaining liver tissues were flash-frozen in liquid nitrogen and stored at -80 °C until analysis.

Carbon tetrachloride dissolved in mineral oil was injected intra peritoneally into the mice. In short-term experiments, mice were injected with CCl4 in mineral oil (1:5 dilution) and killed after 24, 36, 48 and 72 hours of injection, then blood and liver were collected. In long-term experiments, mice were injected with CCl4 in mineral oil (1:10 dilution) for 6 weeks and killed, blood and liver were collected 2 days (will be 6 weeks TP), 5 days (6 weeks+5d) and 10 days (6 weeks+10d) after the last injection. Some mice were injected intra peritoneally with 0.35µg/ g mouse Fas antibody (Jo-2) (BD Pharmingen, San Diego, CA) as positive controls for apoptotic cell death and sacrificed after 6 hours.

2.2 Genotyping:

2.2.1 DNA Extraction from mouse tails:

Wild type, Bid$^{-/-}$ mice were identified from the mouse colony by PCR amplification method. The DNA was extracted from the mouse tails by a standard method. In brief, a small piece of tissue was cut from the tip of the mouse tail and digested in 500µl of lysis buffer

(10mM NaCl + 10mM Tris-HCl (pH8.0) + 25mM EDTA pH8.0 + 0.5% SDS; 64µl of proteinase K was added to 10ml of this buffer) overnight with continuous shaking at 56ºC. Then 50µl of saturated NaCl was added to it and continuously but gently shaken for 1 min at room temperature and then centrifuged at 13,200 rpm for 10 min. To the supernatant 200µl of iso-propanol was added (for DNA precipitation) and gently shaken by inverting the eppendorf tube for 1 min and then centrifuged at 13,200 rpm for 10 min. The supernatant was discarded and the DNA pellet was washed twice with ice cold 70% ethanol and centrifuged at 13,200 rpm for 5 min. The DNA pellet was air dried for 5 min and dissolved in 50µl of TE buffer (10mM Tris-HCl (pH7.6), 10mM EDTA).

Primers and the reaction conditions used for genotyping are as follows:

2.2.2 Genotyping for Bid mice:

17B14: 5'- CCG AAA TGT CCC ATA AGA G -3'

17B12: 5'- GAG ATG GAC CAC AAC ATC -3'

JR23: 5'- TGC TAC TTC CAT TTG TCA CGT CCT -3'

The PCR reaction mixture contains 1µl DNA+ 1µl 17B14 primer (20pmol/µl) + 1µl JR23 primer (20pmol/µl) + 1µl 17B12 primer (6pmol/µl) + 2.5µl 10x reaction buffer (17.5mM) + 2µl dNTP mix (2.5mM) + 1.6µl MgCl2 (25mM) + 0.2µl Taq polymerase and 15µl PCR grade distilled water. The PCR cycle profile is as follows: initial denaturation at 94ºC for 1 min, again at 94º for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min. Thirty cycles of PCR amplification were performed and the PCR products were run on 1- 1.5% agarose gel and visualized with ethidium bromide (25µl of 1mg/ml conc. for 100ml of agarose gel). The primers amplify a fragment of 130 bp for *wild type* and 350 bp for $Bid^{-/-}$ and both the fragments for $Bid^{+/-}$**.**

2.2.3 Genotyping for c-Jun mice:

Lox5: 5'- CTC ATA CCA GTT CGC ACA GGC GGC 3'

Lox6: 5'- CCG CTA GCA CTC ACG TTG GTA GGC 3'

Flox2 5'- CAG GGC GGT GTG TCA CTG AGC T 3'

The PCR reaction mixture contains 2µl DNA+ 1µl Lox5 primer (20pmol/µl) + 1µl Lox6 primer (20pmol/µl) + 1µl Flox2 primer (20pmol/µl) + 5µl 5x reaction buffer (17.5mM) + 2µl dNTP mix (2mM) + 0.4µl Taq polymerase and 9.1µl PCR grade distilled water. The PCR cycle profile is as follows: initial denaturation at 94ºC for 3 min, again at 94º for 30 sec, annealing at 64ºC for 30 sec, extension at 72ºC for 30 sec and 72°C for 5 min. Thirty cycles of PCR amplification were performed and the PCR products were run on 1- 1.5% agarose gel

and visualized with ethidium bromide (25µl of 1mg/ml conc. for 100ml of agarose gel). The primers amplify a fragment of 300 bp for *wild type,* the *deleted C-Jun* fragment is 550 bp and the *floxed c-Jun* is 340 bp**.**

2.3 Serum measurements:

Blood is collected by retro orbital method and spinned at 10,000 rpm for 3 min. After spinning, plasma was recovered and stored at -80°C until used for determination of alanine aminotransferase (ALT/GOT), aspartate aminotransferase (AST/GPT), bilirubin, glucose as markers of liver damage.

2.4 Histology:

2.4.1 Haematoxylin and Eosin (H&E) staining:

Liver issues were fixed in 10% phosphate-buffered formalin, pH 7.4, dehydrated in 100% ethanol, and embedded in paraffin wax at 58°C. Five-micron slides were rehydrated and stained with haematoxylin/eosin (H&E) staining in order to assess the liver damage. First deparaffinised in xylene 2 times with 5 min interval and rehydrated with 100% ethanol 2 times with 3 min interval, with 95% ethanol for 1 min and finally rinsed in distilled water. Slides then stained in haematoxylin for 15 sec and rinsed in tap warm water for 5 min to allow the stain to develop, and shortly rinsed in deionised water. Slides were later stained with acidified eosin for 2 min, rinsed shortly with deionised water and then rehydrated with a series of ethanol in descending order, finally cleared with xylene and mounted the slides using a xylene based mounting medium. Nuclei stained violet and cytoplasm stained pink.

2.4.2 Sirius red staining:

Sirius red staining is used to detect liver fibrosis. 5 microns thick liver slides were deparaffinised in xylene 2 times with 5 min interval and hydrate with 100% ethanol 2 times with 3 min interval and hydrate with 95% ethanol for 1 min and finally rinse in distilled water. Nuclei were stained with weigert´s haematoxylin for seconds and slides were rinsed in distilled water for 10 minutes. Then the slides were stained in picro-sirius red (solution A- picro-sirius red 0,5g in 500ml of saturated aqueous solution of picric acid, added a little solid picric acid to ensure saturation) for one hour, washed in two changes of acidified water (solution B- acidified water: add 5ml acetic acid to 1 litre of water) with 5 min interval, dehydrated in three changes of 100% ethanol and finally cleared in xylem and mount in resinous medium. In this staining nuclei stain black; muscle, red blood cells, fibrin stained red

and cytoplasm stain yellow.

2.4.3 Masson's trichome staining:

This method is used for the detection of collagen fibers in tissues. After deparaffinisation of 5 microns of liver section with xylene, rehydrated with a series of ethanol and stained with haematoxylin 1 min (prepare solution A + B direct before using from **Sigma**), rinsed with water for 5 min. Then slides were stained with Ponceau solution for 5 min, rinsed shortly with 0,5% glacial acetic acid up to 1 min, stained with Orange G solution for 2 min, and were again rinsed shortly in 0,5% glacial acetic acid. Slides were then stained with Light green solution for 3 – 5 min, again rinsed with up to 1 min in 0,5% glacial acetic acid and then proceeded with ascending alcohol sequence, finally cleared in xylene and also mounted with resinous mounting medium. The collagen fibers will be stained blue and the nuclei will be stained black and the background is stained red.

2.5 Immunohistochemistry:

2.5.1 TUNEL assay as apoptosis marker:

After deparaffinisation of 5 microns of liver section with xylene, rehydrated with a series of ethanol, peroxidises were blocked in 3% H_2O_2 (10 ml 30% H_2O_2 in 90 ml Ch_3OH) for 10 min, washed in distilled water twice with 5 min interval. Then the tissue is permeabilised by heating in 0,01 M citrate buffer (pH 6) in a plastic cuvette for approximately 3 min at 750W and 15 min at 150W in a microwave. Slides were then allowed to cool down for 10 min. After washing twice with 1x PBS, tissue was permeabilised with proteinase K solution (20 µg/ml in PBS) for 30 min at room temperature, again rinsed twice with 1x PBS. Then the section was incubated in 4% paraformaldehyde (in PBS) for 1 hour, again rinsed twice with 1x PBS and then section was incubated with 0.1% Sodium citrate, 0.1% Triton X for 30 min (100ml: 0.1g Sodium citrate, 100µl Triton X, better if prepare fresh every time), rinsed twice with 1x PBS. Later the section was incubated with Tunel reaction for 1.5 hour (1:10 dilution; 20-40 µl /slide), rinsed twice with 1x PBS and finally mounted with aqueous mounting medium.

2.5.2. Cleaved caspase 3 staining as a marker of apoptosis:

Immunohistochemistry was performed to detect cleaved caspase-3 in liver slides. Frozen slides were first fixed with 1:1 ratio acetone/methanol at -20° C for 10 min. Then washed with 1x PBS twice with 5 min interval, then incubated with cleaved caspase-3

primary antibody in 1:100 dilution in 3% BSA with PBS-T at 37° C for 2 hours. After the incubation slides were washed twice with PBS-T with 5 min interval. Slides then incubated with rabbit alexaflour 488 fluorescence secondary antibody (1:200 dilution) at 37° C for 1 hour. Then again washing steps carried out with PBS-T then let the slides dried shortly in dark and mounted with aqueous mounting medium having DAPI. Staining was observed under the microscope at Original magnification X100 and representative pictures were taken.

2.5.3 BrdU staining as proliferation marker:

In all experiment, especially in short-term experiments, mice received BrdU either 2 and 24 hours before (in Bid BDL project) or 2 hours (in Bid CCl4 short-term experiments) before harvest. In Bid CCl4 long-term experiments, mice were fed with BrdU containing water 3 days before harvest.

To detect BrdU labeled nuclei, slides were deparaffinized and rehydrated in series of ethanol and permeabilised in 1mM sodium citrate buffer by heating at boiling temperature, 95^{o} C for 30 minutes in water bath. After that the slides were taken out from water bath and allowed to cool down at room temperature for 40 minutes. The slides were then washed in PBS twice and denatured in 2N HCL (83 ml H_20 + 16 ml 37%HCl) for 1 hour at room temperature. After washing 3 times with distilled water, slides were blocked in 3% H_20_2/methanol (8 ml 30% H_20_2 + 72 ml Ch_3OH) for 15 min. In order to block endogenous peroxidase activity, the slides were again washed twice with water. Then the section blocked with 10% goat serum in PBS-Tween for 30 min, later incubated in primary antibody in 1:200 dilution in 5% goat serum in PBS-Tween overnight at 4^{o} C. Next day, after washing the slides in PBS-T secondary antibody (anti-mouse) was added in 1:200 dilution in 5% goat serum in PBS-T for 30 min, again rinsed with buffer, added ABC (from VECTOR) for 20-30 min at room temperature, after washing in buffer, added AEC till got desired colour (more than 5 min), rinsed well in water, counterstained with haematoxylin and mounted with aqueous mounting medium. The number of BrdU positive cells was counted in 200x magnetic field per mouse.

2.5.4 Ki 67 staining as proliferation marker:

After deparaffinisation, and rehydration with a series of ethanol steps, the slides were rinsed in distilled water for 5 min. Then antigen retrieval was performed by heating the slides in EDTA Antigen Retrieval buffer (1mM EDTA, 10mM Tris Base, 0,05% Tween) at 95^{o} C for 30 minutes in a water bath. After that the slides were taken out of the water bath, allowed

to cool down at room temperature for 40 minutes, and rinsed shortly in water. Then placed in Methanol /H_2O_2 blocking mixture (90ml Methanol + 10ml H_2O_2) for 10 min to blocking endogenous peroxidises, and were again rinsed shortly in water. Then blocked in avidin and biotin blocking 15 min each separately, with PBS-T washings in between, again blocked in 5% Goat Serum in PBS-T for 1 hour. Primary antibody was applied to slides in 1:1000 dilution 2% goat serum in PBS-T at 4° C overnight. Next day slides were rinsed with PBS-T twice with 5 min interval each and applied broad spectrum antibody plus for 30 min at room temperature, again rinsed with buffer, applied HRP-streptavidin plus for 15 min at room temperature. After washing twice with the buffer AEC chromogen is applied to the slides approximately 5 to 10 min until they develop colour, then counter stained with Mayer's Haematoxylin for about 15 sec. After rinsing in water, slides were mounted with aqueous mounting medium.

2.5.5 Alpha-sma staining as a fibrosis marker:

After deparaffinisation with xylene and rehydration with a series of ethanol, antigen retrieval carried out by heating the slides in 0.1mM sodium citrate buffer at 600W for 3 min and at 150W for 10 min in a microwave. After washing twice with PBS for 5 min, slides incubated with a-sma primary antibody from dakocytomation in 3% BSA in PBS-T in 1:100 dilution overnight at 4° C. After washing twice with PBS, slides then incubated with alexa 488 fluorescent secondary antibody, 1:200 dilution in 3% BSA in PBS-T for 1 hour in dark, after washing the slides twice with PBS, let them dried and mounted with aqueous mounting medium having DAPI, to stain nuclear material.

2.5.6 Naphthol AS-D chloroacetate esterase staining for neutrophills:

After deparaffinisation, rehydration with a series of ethanol steps, slides were washed shortly with distilled water. After that the slides were fixed with citrate-acetone-formaldehyde solution for 30 sec at room temperature. After fixation, rinsed shortly with distilled water, slides were incubated for 2 to 3 hours in a coplin jar having the solution mixture of 1ml Naphthol AS-D chloroacetate, 1ml sodium nitrite, 1ml fast red violet LB base solution and 5ml Trizmal prepared in 40ml of distilled water. After the incubation, slides were removed from the jar and rinsed thoroughly in distilled water for atleast 2 min. Then counterstained with haematoxylin and evaluated microscopically after mounting with aqueous mounting medium. In this staining neutrophills stained pink to violet colour and they can be distinguished from the other cells, hepatocytes with their morphology.

2.5.7 CD11b staining for neutrophill:

This staining carried out on frozen slides of liver. The slides were fixed in 1:1 ratio acetone/ methanol for 10 min at -20° C; the slides were then rinsed in TBS-T twice with 2 min interval. The slides were blocked with 5% normal horse serum in TBS-T for 30 minutes at room temperature and incubated with rat CD11b primary antibody in TBS-T with 5% normal horse serum at 37°C (1:50 dilution) for 2 hours. After incubation, the slides were rinsed in TBS-T twice, then applied anti rat fluorescence secondary antibody (1:100 dilution) in TBS-T with 5% normal horse serum and incubated at room temperature for 1 hour, then rinsed the slides in TBS-T twice and finally mounted in an aqueous mounting medium with DAPI.

2.5.8 CD68 staining for Kupffer cells:

Frozen tissue slides were fixed with acetone/methanol in 1:1 ratio for 10 min at -20^{o} C, after that rinsed in TBS-T twice and incubated with rat CD68 primary antibody (1:100 dilution) in 3% BSA in TBS-T at 37^{o} C for 2 hours. After washing twice with TBS-T, slides were incubated with anti rat fluorescence secondary antibody (1:200 dilution) at 37^{o} C for 1 hour and after incubation washed twice with buffer, dried the slides shortly and mounted using aqueous mounting medium with DAPI.

2.6. Western blot:

2.6.1 Protein preparation:

Protein lysate was prepared from the liver tissue and from cultured hepatocytes by homogenisation in readymade cell lysis buffer (from cell signalling) having "complete" (a protease inhibitor) in it. After the homogenisation, three times freeze & thaw cycles carried out to break down the cellular membranes. The lysate then centrifuged at 13,200 rpm for 10 min and collected the supernatant. The protein was quantified by using Bio-rad reagent with photometer. 100µg of protein is treated with loading buffer (0.5MTris HCl pH6.8, SDS powder-640 mg, 100% glycerol-3.2ml, ß-mecaptoethanol-1.6ml, bromophenopl blue - 0.001gm dissolved in distilled water 2.14ml) and heated at 95°C for 10 min.

2.6.2 Protein separation through SDS-PAGE and western blotting:

After loading the protein lysate in to the gels, they were allowed to run in pre-chilled 1x running buffer (Tris – 3.03 gm, glycine – 14.42 gm and 10 ml 10% SDS makeup to 1 litre with millipore water) at 20mA/gel (constant) approximately 1-2 h in an ice bucket. After

running the gels, they were transferred to PVDF membrane (Millipore, Bedford, MA) in pre-chilled 1x transfer buffer (Tris – 3.03 gm, glycine – 14.42 gm makeup to 1 litre with millipore water) at 100 V for 60 min with ice pack in cold room, continuous stirring is carried out in the blotting unit during transfer. After that the membrane washed in TBS-T (1M Tris pH 7.5 – 50ml, NaCl- 8.76 gm and 1 ml Tween 20 make upto1 litre with Millipore water) and blocked the membrane in 10 ml of 5% non fat dry milk powder in 1x TBS-T for 30 min on shaker at room temperature. After that the membrane incubated with correspond-ding primary antibody in different dilutions depends on the antibody in 10 ml volume on rocker overnight at 4^0 C. After incubation with primary antibody, on next day, the membrane washed twice with 1x TBS-T with 15 min interval. After that the membrane incubated with corresponding secondary antibody depends on the host in which primary antibody developed in 1:3000 dilution. The membrane was rinsed twice with 1x TBS-T with 15 min interval. The membrane was then developed with chemiluminescence reagent on the chemiluminescence films in the developing machine.

2.7 Real-time RT PCR for mRNA level expressions:

2.7.1 Isolation of total RNA using Trizol:

Liver tissue (20-30 mg) was homogenized in 500µl of Trizol reagent using homogenizer for about some seconds and 100µl of chloroform was added and placed at room temperature for 5 min. The lysate then centrifuged at 12,000 g for 15 min at 4° C. After centrifugation upper aqueous layer was collected, to this 250µl of iso-propanol was added and mixed vigorously. After incubation at room temperature for 10 min, it was spinned again at 12,000 g for 10 min at 4° C. The RNA should be in pellet form, so the pellet was washed with 500µl of 75% ethanol by vortexing, and spinned at 7500 g for 5 min at 4° C. Then the pellet was briefly dried and, resuspended the pellet in RNase free distilled water.

2.7.2 cDNA synthesis and real time RT PCR:

RNA samples of 4 mice in each group were pooled and 4 µg RNA from every pool was used to synthesize cDNA with the superscript ^{TM} II first-strand synthesis (Invitrogen, Carlsbad, Ca). 20 ng of cDNA was amplified in a total reaction volume of 25 µl in an Applied Biosystems 7300. Conditions used for the PCR was first, denaturation at 95^o C for 10 sec, annealing at 52.8^o C for 30 sec and extension at 72^o C for 30 sec, 45 cycles .

	Gene	***Primer sequences***
1.	a-sma (forward)	ACAGCCCTCGCACCC A
	a-sma (reverse)	GCCACCGATCCAGACAGAGT

2.	Tgfß-1 (forward)	AGAGGTCACCCGCGTGCTAA
	Tgfß-1 (reverse)	TCCCGAATGTCTGACGTATTGA
3.	Col a1 (forward)	TCCGGCTCCTGCTCCTCTTA
	Col a1 (reverse)	GTATGCAGCTGACTTCAGGGATGT
4.	Timp1 (forward)	TCCTCTTGTTGCTATCACTGATAGCTT
	Timp1 (reverse)	CGCTGGTATAAGGTGGTCTCGTT
5.	Mmp3 (forward)	GATGGACGATGGACAGAGGATG
	Mmp3 (reverse)	AGGGAGTGGCCAAGTTCATG
6.	Mmp13 (forward)	GGAAGACCCTCTTCTTCTCT
	Mmp13 (reverse)	TCATAGACAGCATCTACTTTGTT
7.	Tnf-Alpha (forward)	ATGAGCACAGAAAGCATGATC
	Tnf-Alpha (reverse)	TACAGGCTTGTCACTCGAATT
8.	KC (forward)	GGATTCACCTCAAGAACATCCAGAG
	KC (reverse)	CACCCTTCTACTAGCACAGTGGTTG
9.	MCP1 (forward)	TCCATGCAGGTCCCTGTCATGCTT
	MCP1 (reverse)	CTAGTTCACTGTCACACTGGTC
10.	MIP1 (forward)	CCTCAA CGGAAGAACCAAAGAG
	MIP1 (reverse)	CTCAGACAGCGAGGCACATC
11.	IL-6 (forward)	CCACTTCACAAGTCGGAGGCTTA
	IL-6 (reverse)	GCAAGTGCATCATCGTTGTTCATAC
12.	ß2mg (forward)	CTGATACATACGCCTGCAGAGTTAA
	ß2mg (reverse)	ATGAATCTTCAGAGCATCATGAT
13.	Gapdh (forward)	GGATGCAGGGATGATGTTC
	Gapdh (reverse)	TGCACCACCAACTGCTTAG

2.8. Caspase-3 activity assay:

Protein lysate was prepared from the livers of both *wild type* and *Bid*$^{-/-}$ mice undergone either BDL or CCl4 treatment and also the treated hepatocytes using 1x cell lysis buffer from BD biosciences. After three times freeze thaw cycles carried out and centrifuged at 13,200 rpm for 10 min and the supernatant was collected. 50µg of protein lysate was used to measure the caspase-3 activity. To the 50µg protein, 200µl of assay buffer (40mmol HEPES, 20% Glycerol, 4mmol DTT, pH 7.4), 5µl of the Ac-DEVC-AMD Caspase-3 (CPP32) fluorogenic Substrate was added and with or without Ac-DEVD-CHO Caspase-3 (CCP32) inhibitor, incubated at 37° C for 1 hour and the fluorescence of samples was measured in top read using

in 96-Well-Plate (black bottom) with Elisa reader at excitation wavelength 380nm and emission wavelength 450nm.

2.9 Measurement of Hepatic Hydroxyproline Content:

Total hepatic hydroxyproline levels at different time points after BDL and CCl4 treatment was determined in the hydrolysates of liver samples. Briefly, precisely weighed liver tissue samples 100 mg were homogenized in distilled H_2O. The homogenates were hydrolyzed in 10 N HCl by incubation at 110° C for 18 hours. The hydrolysates were dried by speed vacuum centrifugation over 3 to 5 hours and redissolved in a buffer containing 0.2 mol/L citric acid, 0.2 mol/L glacial acetic acid, 0.4 mol/L sodium acetate, and 0.85 mol/L sodium hydroxide, pH 6.0. Hydroxyproline levels in the hydrolysates were biochemically measured using photometer.

3.0 Cell culture method:

3.1 Isolation of primary hepatocytes:

Mice were anaesthetized, the abdomen was opened and the perfusion of liver carried out through hepatic portal vein shortly few minutes with solution-1 (EBSS without Ca and Mg [Gibco 310-4150AJ] with 0.5 mM EGTA [Sigma 4378]), and then few minutes with solution-2 (EBSS with Ca and Mg [Gibco 310-4010 G], plus 10mm HEPES, pH 7.4 [Sigma]) and finally with solution-3 having collagenase (Blendzyme) to digest the liver completely. Once the liver digested fully, liver was taken out with scissors, tiered and cut into small pieces and was suspended into the DMEM medium having 10% FBS. The cell suspension was then passed through 100 µM nylon mesh into 50ml falcon tube in order to get rid of the other liver cells. The cell suspension was centrifuged at 50 g for 3 min, and was repeated twice until we got a clear supernatant. Then the cells were counted using trypon blue exclusion assay. Nearly 1.5 million cells were plated in DMEM medium with 10% FBS and penicillin and streptomycin in a pre collagen coated 60mm culture dish.

3.2 Treatment of hepatocytes:

Hepatocytes were treated with 100 µM and 300 µM concentrations of DCA (Deoxy cholic acid) and GCDC (glycol-cheno deoxy cholic acid) for 6 hours in DMEM medium without FBS and with penicillin and streptomycin. After the treatment the cells were pelleted by spinning at 1500 rpm for 7 min and washed with sterile PBS. The cells were lysed in cell lysis buffer first, after wards three freeze thaw cycles was carried out and finally the lysate

was centrifuged at 13,200 rpm for 10 min in order to get total protein. For isolation of mitochondrial protein a special homogenizer called dounce homogenizer was used.

3.3 Apoptosis assay on hepatocytes- TUNEL staining:

After treatment the cells were washed with sterile PBS once and were fixed in 4% paraformaldehyde for 30 min at room temperature. After the fixation, cells were washed with PBS and permeabilised with 0,1% sodium citrate having 0,1% Triton X-100 for 5 min on ice. Then Tunel reaction mixture was added to the cells and incubated in dark at 37^0 C for 1 hour. The cells were then washed with PBS-T twice with 5 min interval and were dried shortly in dark and mounted with aqueous mounting medium having DAPI. Then cells were observed and the representative pictures were saved in Original magnification X100.

4.0 Antibodies	**Company**
Actin	Santa Cruz
Alpha- sma- anti mouse	Dakocytomation
BrdU anti mouse antibody	Amersham
BrdU anti mouse antibody	BD pharmingen
Bcl-x anti rabbit	Santacruz
Bax- anti rabbit	Santacruz
Bid- anti goat	R&D
Caspase-9- anti rabbit	Cell signaling
CD11b-anti-rat	BD Pharmingen
CD68-anti-rat	Allexis
cFlip- anti rabbit	Sressgen
C-Jun- anti rabbit	Cell signaling
Cleaved caspase-3- anti rabbit	Cell signaling
Cyclin A- anti rabbit	Santacruz
Cyclin B1- anti rabbit	Santcruz
Cyclin D1- anti mouse	BD pharmingen
Cyclin E- anti rabbit	Sanatcruz
Fas- anti rabbit	Santacruz
Mcl-1- anti rabbit	Rockland
Phospho C-Jun- anti rabbit	Cell signaling
Phospho ERK- anti rabbit	Cell signaling

Phospho JNK- anti rabbit	Cell signaling
Phospho p38- anti rabbit	Cell signaling
Phospho stat3- anti rabbit	Cell signaling
p21- anti goat	Santacruz
Stat3- anti rabbit	Cell signaling

4.1 Cell culture reagents

Advanced DMEM medium	Gibco
Collagen A	Biochrome
Fetal bovine serum	TAA
PBS (phosphate buffered saline)	Gibco
Pencillin/streptomycine	Gibco
Trypsin/EDTA solution	TAA

4.2 Chemicals

Agarose (Electrophoresis grade)	Invitrogen
Ammonium chloride	Sigma
Ammonium per sulphate	Fluka
Aqueous based mounting medium	Zymed
5’-bromo-2’-deoxyuridine (BrdU)	Sigma
Bromophenol blue	Sigma
BSA (bovine serum albumin)	Sigma
Chemiluminesence Film	Amersham
Chemiluminesence reagent	PERKin elmer
Complete mini (protein stabilizer)	Roche
Chloroform	J.T.Baker
Citric acid	Merck
Deoxycholic acid	Sigma
dNTP´s	Invitrogen
EBSS with/without Ca & Mg	Gibco
EDTA (N, N, N’, N’-Ethylenediaminotetraacetate)	Sigma
Ethanol	J.T.Baker
Ethidium bromide	Sigma
Formaldehyde	Merck

Hemalum solution	Merck
HEPES buffer	Sigma
Hydrochloric acid	Merck
Hydrogen peroxide	Sigma
Isopropanol	J.T.Baker
Mayer´s Haematoxylin solution	Sigma
Methanol	J.T.Baker
Mounting medium with DAPI	Vectashield
NNN´N´ tetramethylethylenediamine (TEMED)	Sigma
Paraformaldehyde	Merck
Potassium bicarbonate	Sigma
Sodium hydroxide	Merck
Sodium chloride	Merck
Sodium dodecyl sulphate (SDS)	Sigma
Tris (Tris-(hydroxymethyl)-aminomethane)	Invitrogen
Tween 20	Sigma
Triton 100	Sigma

4.3 Enzymes, DNA ladder and protein ladder

Proteinase K	Merck
RNase A	Sigma
Taq DNA polymerase -	Go taq Promega
DNA marker-	1kb ladder Life technology
Precision plus protein ladder (Dual color)	Biorad

4.4 Laboratory equipment

100µm filter	Nalgene
22mm cover slips	Menzel-Glaser
24 x 50mm cover slips	Menzel-Glaser
76 x 26mm Super Frost plus – Glass Slides	Menzel-Glaser
Desktop centrifuge	Eppendorf
Electrophoresis-Apparatus	Labtech,Pharmacia Biotech
Electrophoresis apparatus for PAGE	Biorad
Fluorescence microscope	Olympus
Gel doc	Syngene

Heat block/Thermo mixer	Eppendorf
Hyper cassette	Amersham
Microscope	Olympus
Microtome	Leica
Neubaur chamber	Marienfeld
PCR master cycler	Eppendorf
PCR soft tubes	Biozym
Power-Supply	Biometra
Spectrophotometer	Eppendorf
Vortexer	Omnilab
Western blotting chamber	Biorad

4.5 Kits

Avidin /biotin blocking kit	Vectashield
In situ cell death detection kit	Roche
Super script first strand cDNA synthesis kit	Invitrogen
High fidelity cDNA synthesis kit	Roche

***Statistical analysis*:** Data are expressed as mean ± SD determined by 1-way analysis of variance followed by Student T test to determine significance. A p value below 0.05 was considered significant. 4 to 5 mice in each group were analyzed.

5.0 Results:

5.0. The BH3-only protein bid does not mediate death-receptor-induced liver injury in obstructive cholestasis:

5.1 Loss of Bid does not affect liver injury and overall survival of mice following BDL.

Cholestasis was induced in a cohort of experimental mice by surgical bile duct ligation. In *wild type* mice, serum aspartate aminotransferase (AST) and bilirubin levels rapidly increased following BDL. AST levels peaked on day 2-3 and declined thereafter. Bilirubin levels remained highly elevated following BDL (Fig.1A). In contrast to Fas-receptor deficient *lpr* mice, a similar increase in plasma AST activities and bilirubin levels was evident in $Bid^{-/-}$ mice at all time points analyzed (Fig 1 A). Concomitant to biochemical liver injury

parameters, multiple bile infarcts were evident in livers of *wild type* and *Bid*$^{-/-}$ mice three days following BDL (Fig 1B). Hepatocytes in these foci exhibited characteristics of oncotic cell death as previously described. Fewer bile infarcts were detectable in livers of both groups at later time points paralleling declining AST levels. In agreement with the biochemical and histological data, survival curves did not reveal a significant difference between *wild type* and *Bid*$^{-/-}$ mice (Fig. 1C).

5.1 Figure A, B and C

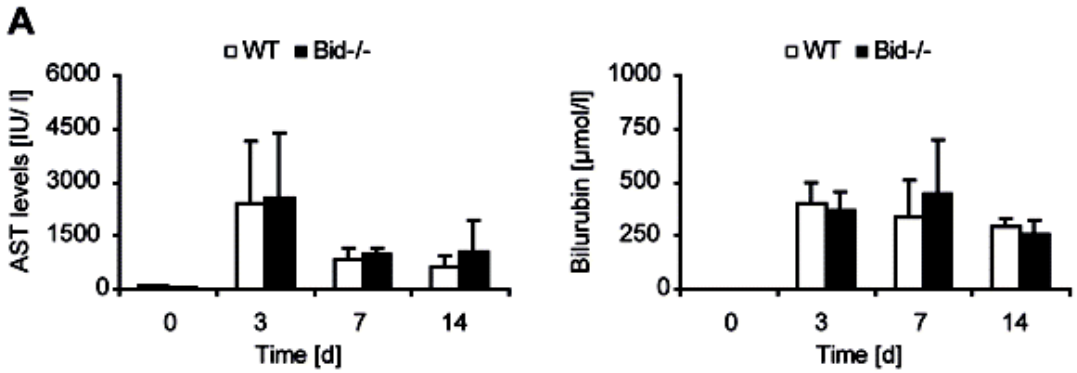
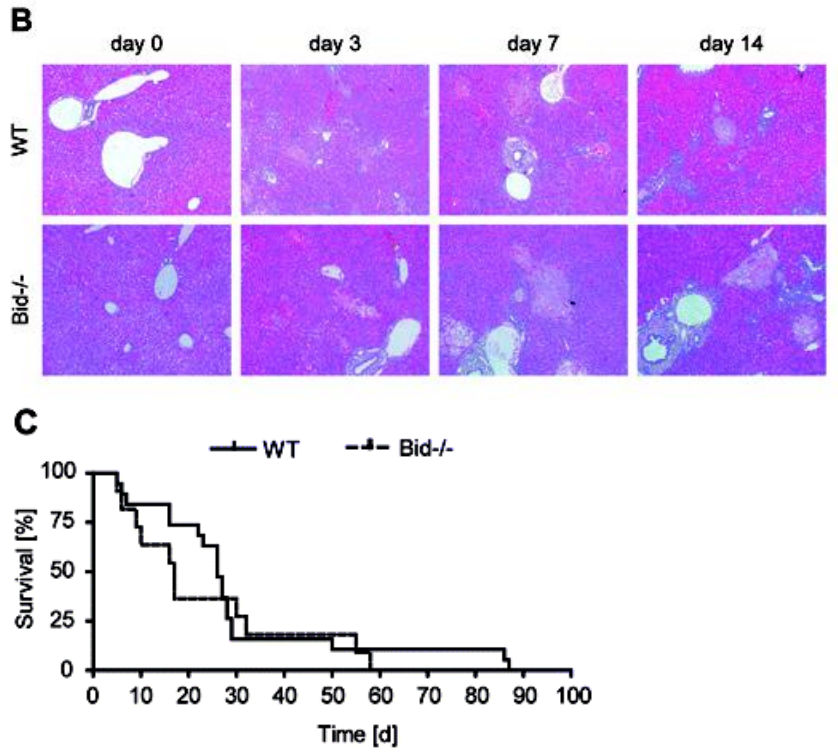


Figure 5.1 (**A**) *Wild type* (WT) and *Bid*$^{-/-}$ mice underwent bile duct ligation and were euthanized after 3, 7 and 14 days. Serum levels of AST and bilirubin as markers of hepatic damage were measured at indicated time points. Mean values and standard deviation are presented for all time points (n=5 in each group).



(**B**) H&E staining revealed profuse liver damage and multiple oncotic bile infarcts in BDL mice. (**C**) Survival of *wild type* and *Bid*$^{-/-}$ mice was recorded following BDL (n= 20 mice in each group).

5.2 Loss of Bid does not affect apoptosis of hepatocytes following BDL mice

In agreement with previous studies, oncotic foci also stained positive with the TUNEL assay (Fig. 5.2 A). Additionally, numerous TUNEL positive cells were disseminated throughout the lobules of BDL livers. However, positive immunoreactivity for cleaved caspase-3 indicative of apoptosis was rarely seen. Consistent with these findings, there was no significant increase in caspase-3 activity in *wild type* mice before or after BDL (Fig. 5.2 B).

5.2 Figure A, B, C and D

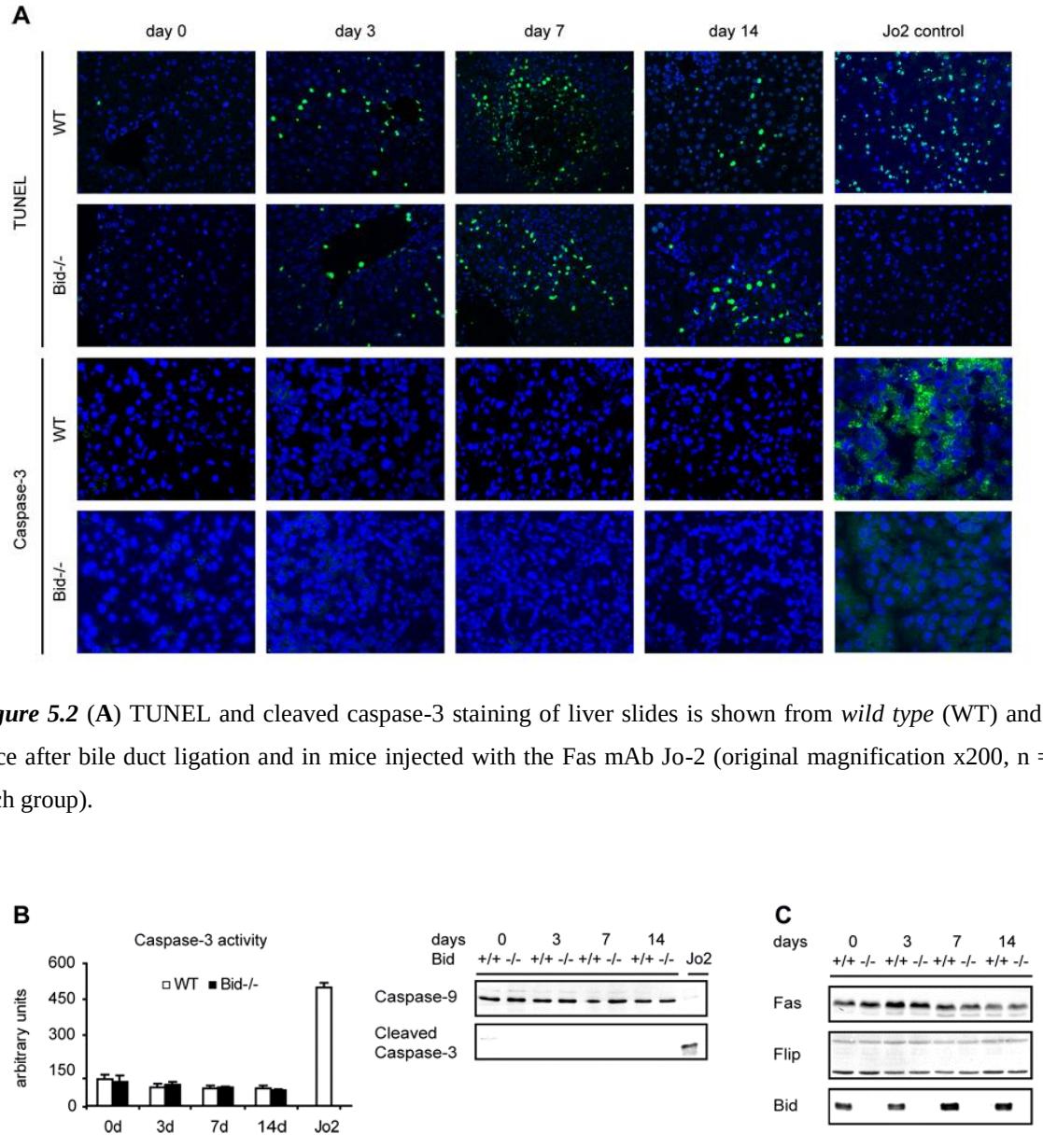


Figure 5.2 (**A**) TUNEL and cleaved caspase-3 staining of liver slides is shown from *wild type* (WT) and *Bid*$^{-/-}$ mice after bile duct ligation and in mice injected with the Fas mAb Jo-2 (original magnification x200, n = 4 in each group).

Figure 5.2 (**B**) Hepatic caspase-3 activity was measured using the CaspACE Assay kit and processing of caspase-9 (loss of full length caspase-9) and caspase-3 (detection of the cleaved fragment) was analyzed by Western blot for all time points before and after BDL and in Jo-2 injected mice as a positive control (+). (**C**) Liver tissues of control and BDL mice were analyzed for total cellular levels of Fas, Flip and Bid (n=5, pooled samples).

Importantly, the same pattern of TUNEL positive cells and cleaved caspase-3 negative cells was evident in *Bid*$^{-/-}$ mice following BDL. In agreement with the enzyme activity data, no significant difference in protein levels of full-length caspase-9 was seen between both groups (Fig. 5.2 B). Furthermore, the cleaved and active fragment of procaspase-3 was not detectable in any BDL group. In contrast, there were multiple TUNEL and caspase-3 positive hepatocytes in livers of Jo-2 treated *wild type* mice (Fig. 5.2 A). Additionally, hepatic caspase-3 activity and processing of caspase-9 and caspase-3 was clearly evident in these apoptosis control mice (Fig. 5.2 B). Protein levels of the Fas receptor were not significantly changed following BDL (Fig. 5.2 C). Additionally, levels of c-Flips/l, a specific inhibitor of caspase-8, remained unchanged in BDL mice. As anticipated, Bid was not detectable in the livers of *Bid*$^{-/-}$ mice. To further analyze the role of Bid in bile acid induced cell death, primary WT and *Bid*$^{-/-}$ hepatocytes were treated with various concentrations (50 – 400 µM) of DCA and GCDC, which have been shown to cause ligand independent activation of the Fas receptor to stimulate cell death in primary hepatocytes (Gupta et al., 2004; Qiao et al., 2002; Qiao et al., 2001). In agreement with previous studies, exposure of primary mouse hepatocytes to low concentrations of bile acids did not significantly increase basal apoptosis within 6 hours (Qiao et al., 2001).

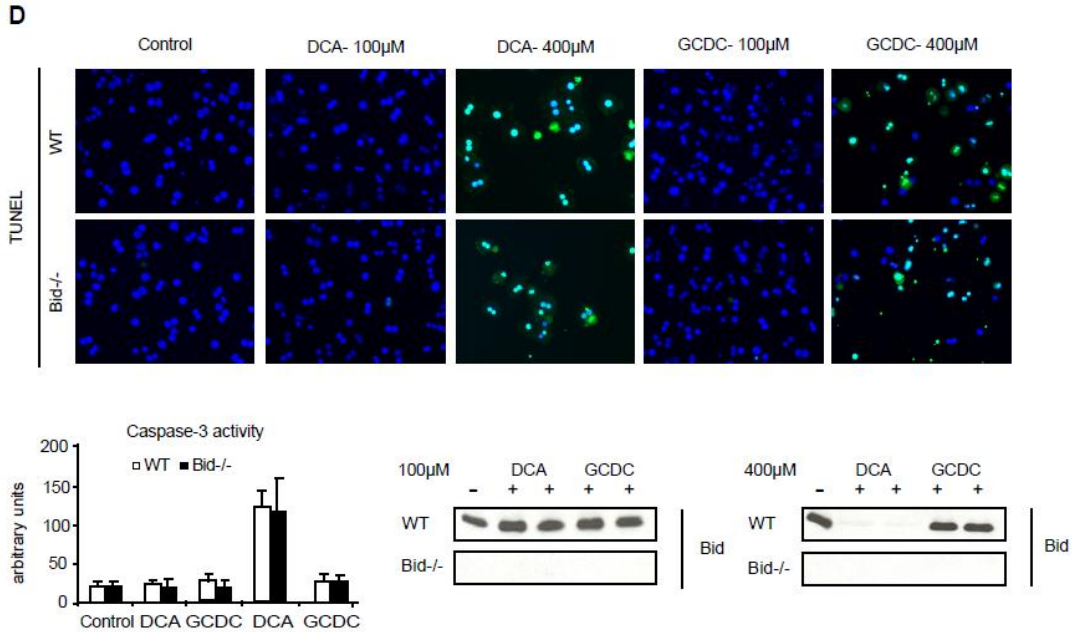


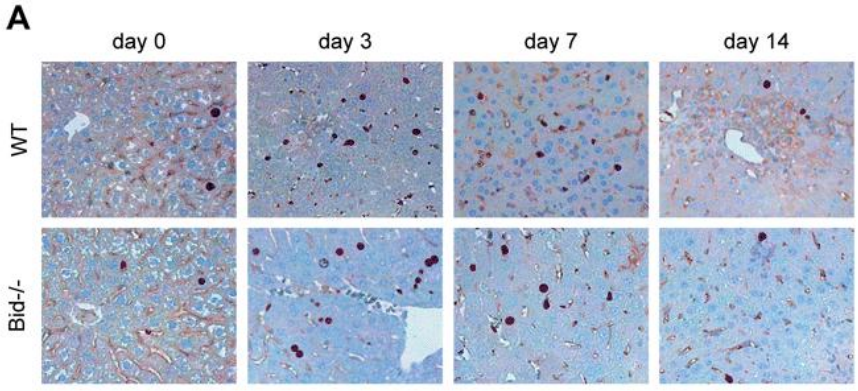
Figure 5.2 (D) caspase-3 activity and TUNEL staining of primary *wild type* and *Bid*$^{-/-}$ hepatocytes treated with 100µM and 400 µM of DCA and GCDC for 6 hours. Processing of Bid (loss of full length protein) was evident in DCA-treated hepatocytes.

Treatment of mouse hepatocytes with higher concentrations of DCA and GCDC however caused significant cell death and multiple TUNEL positive hepatocytes were detectable (Qiao et al., 2001). Processing of Bid and activation of caspase-3 was only evident in DCA-treated hepatocytes suggesting that GCDC induces a caspase independent cell death. Importantly however, there was no significant difference between *wild type* and *Bid*$^{-/-}$ primary hepatocytes exposed to both bile acids confirming that Bid is dispensable for bile acid induced cell death in murine hepatocytes (Fig. 5.2 D).

5.3 Loss of Bid does not affect proliferation of hepatocyte following BDL

Cell death of hepatocytes following BDL induces a regenerative response in the liver (Grambihler et al., 2003). To study whether loss of Bid affects proliferation of hepatocytes during cholestatic liver injury, the number of bromodeoxyuridine (BrdU)-positive hepatocytes was determined 3, 7 and 14 days following BDL (Fig. 5.3 A). Semi quantitative analysis of BrdU-labeled hepatocytes revealed an increase of positive cells with a peak at day 3 (Fig. 5.3 B). A similar number of proliferating hepatocytes was detectable in *wild type* and *Bid*$^{-/-}$ mice. Next, the expression of three key regulators of hepatocellular proliferation was analyzed by western blotting. Levels of Cyclin D rapidly increased in both groups compared with sham-operated controls. In contrast, protein levels of Cyclin E remained almost unchanged at these early time points following BDL (Fig. 5.3 C).

5.3Figure A and B



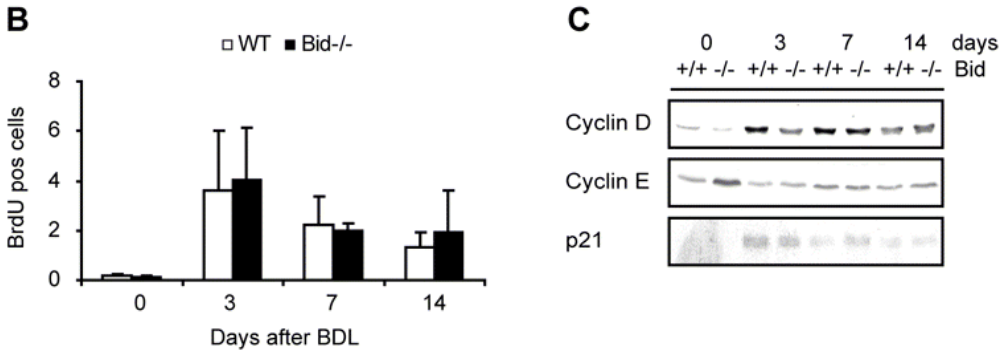


Figure 5.3 (**A, B**) Hepatocyte proliferation was assessed with BrdU immunostaining and BrdU positive cells were quantified (10 microscope fields at Original magnification X200, *wild type* (WT) mice are represented as open bars and *Bid*$^{-/-}$ mice as closed bars, n=5). (**C**) Immunoblots were performed for Cyclin D and E and p21 (n=5, pooled samples).

5.4 Loss of Bid does not attenuate fibrogenesis following BDL

Complete obstruction of the common bile duct results in the rapid development of liver fibrosis. Following BDL, fibrotic lesions mainly occur around the bile ducts. To investigate the role of Bid in liver fibrogenesis, Sirius red and Masson's trichrome staining was performed with liver slides 3, 7 and 14 days after BDL. In *wild type* BDL mice extensive peribiliary and slight interstitial collagen deposition was evident (Fig. 4A). Several lines of evidence suggest that alpha-smooth muscle actin (a-SMA)-positive myofibroblasts are the main effector cells responsible for the overproduction of matrix components in fibrotic liver. Accordingly, multiple SMA positive cells became evident shortly after BDL (Fig. 4A).

5.4 Figure A

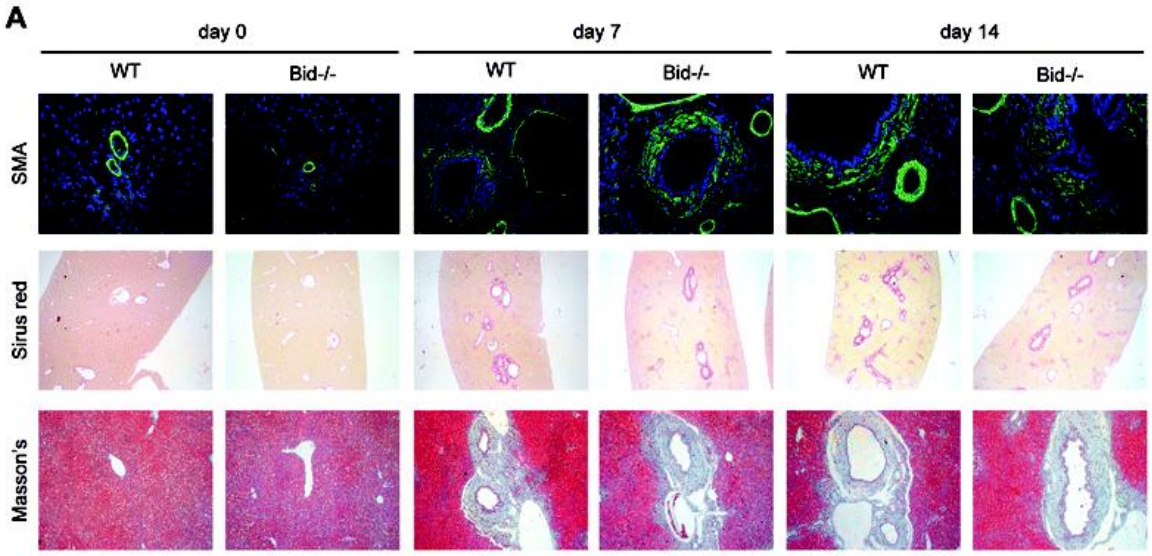


Figure 5.4 **(A)** Liver fibrosis was evaluated by a-SMA, Sirius red, and Masson's trichrome staining 3, 7, and 14 days after BDL (Original magnification X200).

Overall however, there was no obvious difference for collagen deposition and stellate cell activation between *wild type* and *Bid*$^{-/-}$ mice at any time point analyzed. The degree of fibrosis was further quantified by measuring the content of hydroxyproline, which is almost exclusively found in collagen in animal tissues, in hydrolysates extracted from two liver lobes of mice 7 days following BDL. Hydroxyproline content was significantly increased in liver extracts of BDL mice compared with controls (*wild type* day 0: 14.36 + 4.6 (lobe 3), 14.53 + 3.2 (lobe 5); *wild type* day 7: 22.66 + 3.1 (lobe 3; p=0.0052); 20.52 + 3.1 (lobe 5; p=0.01). Interestingly, hydroxyproline content was slightly higher in *Bid*$^{-/-}$ mice (Fig. 5.4 B). Next, mRNA levels of genes implicated in liver fibrosis were quantified by quantitative RT-PCR to determine whether other markers of stellate cell activation were affected by loss of Bid.

5.4 Figure B and C

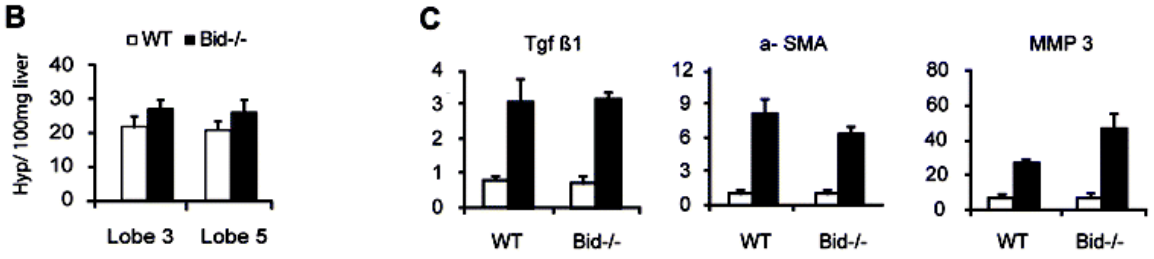


Figure 5.4 **(B)** Hydroxyproline content measured in the right median (#3) and the right lateral (#5) liver lobes 7 days after BDL. **(C)** mRNA levels of Tgf-ß, a-sma and MMP3 were quantified by real-time RT PCR (sham operated mice are represented with open bars and BDL mice with closed bars).

Transforming growth factor (TGF) β, a key fibrogenic cytokine in the liver, a-SMA and matrix metalloproteinase (MMP)-3 were significantly increased three days following BDL in *wild type* mice. Loss of Bid did not significantly affect the transcriptional regulation of any gene analyzed (Fig. 5.4 C).

5.5 Loss of Bid does not affect the inflammatory response following BDL

One important feature of cholestatic liver disease in both humans and rodents is hepatic inflammation. Most prominent among the inflammatory cells are neutrophills, which are recruited within hours into the liver following BDL (Saito and Maher, 2000).

5.5 Figure A and B

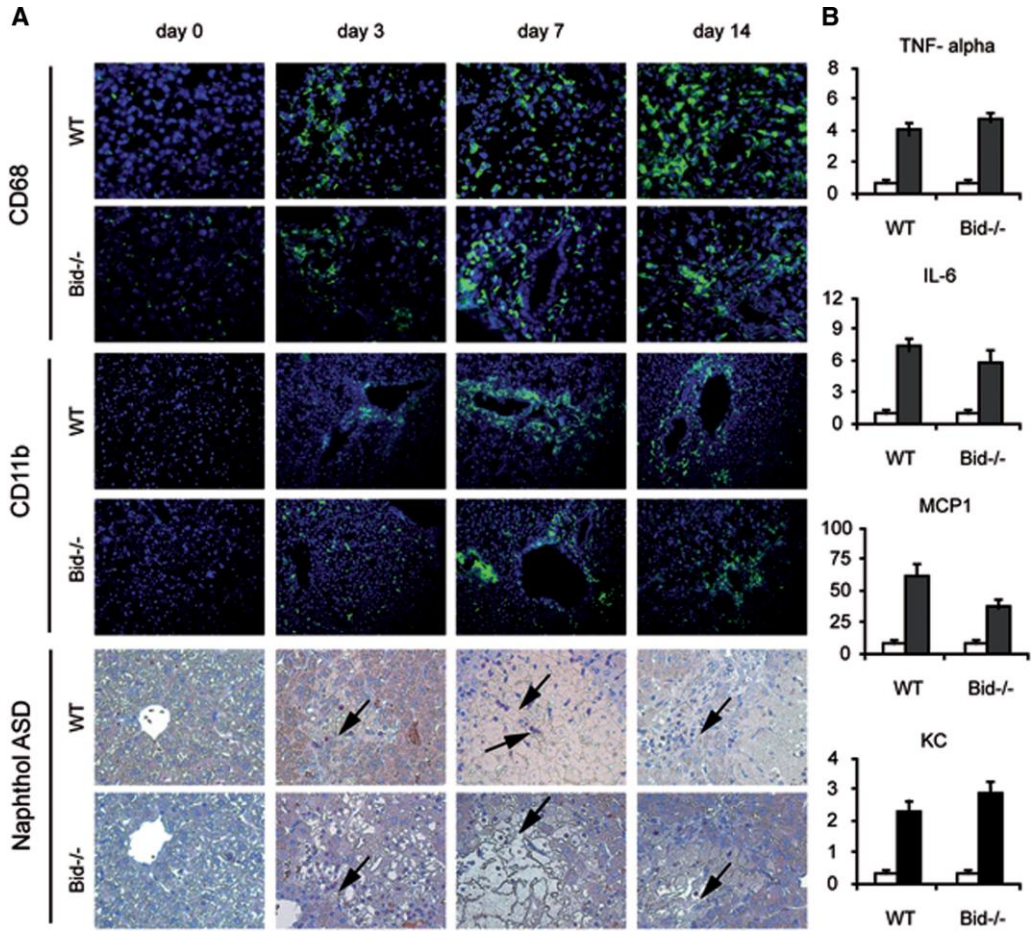


Figure 5.5 (**A**) Liver slides of control and BDL mice were analyzed for CD68 expression and CD11b expression (Original magnification X200). Infiltrating neutrophills were also detected by Naphthol ASD staining. (**B**) Quantitative RT-PCR was performed using pooled RNA extracted from *wild type* (WT) and *Bid*$^{-/-}$ mice before (open bars) and after BDL (closed bars) (n=4). Specific cDNAs of KC, MCP1, IL-6 and TNF-alpha were amplified.

Kupffer cells, resident macrophages of the liver, and other macrophages recruited to the liver, are the major source of inflammatory cytokines and CXC chemokines in the liver that recruit neutrophills to the hepatic parenchyma (Seki et al., 2009). To evaluate the role of Bid for the activation of these inflammatory cells, liver slides were stained with CD68 to label Kupffer cells, and with CD11b and Naphthol ASD to detect neutrophills. In agreement with previous studies multiple neutrophills were observed in the parenchymal tissue of *wild type* mice after BDL (Fig. 5.5 A). Additionally, more Kupffer cells were evident in BDL mice compared with controls. Analysis of *Bid*$^{-/-}$ BDL livers revealed a similar number of neutrophills and Kupffer cells as in BDL *wild type* mice suggesting that Bid does not affect

the recruitment of these cells to the liver. Next, mRNA levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemotactic protein (MCP)-1 and the neutrophill chemokine KC were analyzed by RT-PCR. In agreement with previous studies, mRNA levels of TNF-α and IL-6 were significantly induced in *wild type* mice three days following BDL (Fig. 5B). Furthermore, mRNA levels of KC and MCP-1, potent neutrophill and monocyte chemo attractants, were significantly induced in BDL mice compared with sham-operated controls. In contrast to *lpr* mice, in which the inflammatory response is significantly attenuated, a similar increase was seen in BDL *Bid*$^{-/-}$ mice.

5.6 Bid$^{-/-}$ and *lpr* mice display similar activation of stress kinase pathways following BDL

The data presented above strongly suggest that Bid does not contribute to BDL induced liver injury during cholestasis. However, bile acid induced liver injury was significantly reduced in *lpr* mice compared with *wild type* and *Bid*$^{-/-}$ mice as previously reported (Fig. 5.6 A). We therefore wondered, which other pathways down-stream of Fas-R might play a role in cholestatic liver injury. Recently, it has been shown that bile acids activate various cell signaling cascades including protein kinase C, ERK, Jnk, p38, and PI3 kinase that potentially affect survival of hepatocytes (Canbay et al., 2004). To investigate the contribution of MAPKs in the inflammatory response triggered by Fas, activation of ERK, Jnk, p38 and c-Jun was analyzed in *wild type* and *Bid*$^{-/-}$ mice at different time points following BDL. Additionally, activation of these pathways was analyzed in *wild type* and *lpr* mice three days following BDL. BDL caused a slight activation of ERK, whereas a sustained activation of Jnk and p38 was not detectable (Fig. 5.6 B).

5.6 Figure A

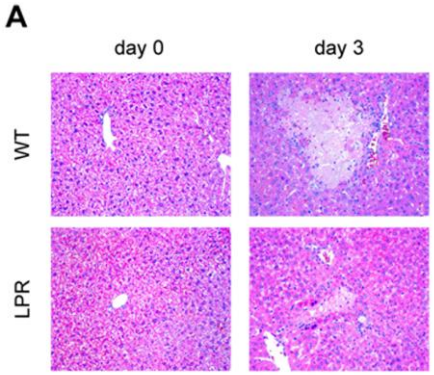


Figure 5.6 (**A**) *Wild type* (WT) and *lpr* mice underwent BDL and were euthanized after 3 days (n=4). Liver injury was analyzed by H&E staining.

There was however no significant difference between *Bid*$^{-/-}$ and *lpr* mice and their respective littermate control mice. Interestingly however, loss of Fas- R markedly reduced the up-regulation of c-Jun in livers of BDL mice (Fig. 5.6 C). To specifically analyze the role of c-Jun in cholestatic liver injury, BDL was performed with conditional *c-Jun*$^{-/-}$ mice. In contrast to *lpr* mice however, a similar number of oncotic foci and similar transaminase levels were evident in *c-Jun*$^{-/-}$ mice suggesting that c-Jun is not a direct downstream effector of Fas-R in BDL induced liver injury (n=6 per group).

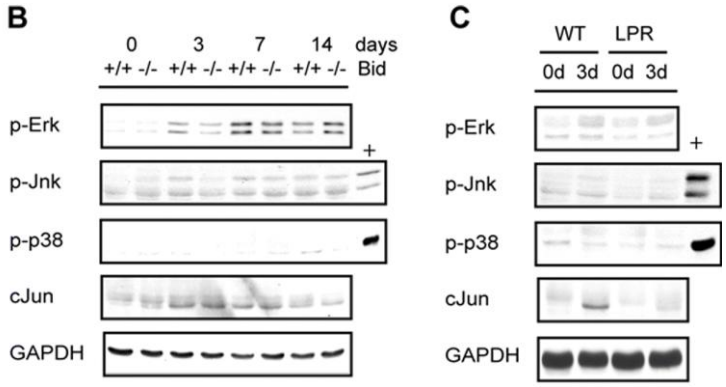


Figure 5.6 (**B**) Western blot of p-ERK, p-Jnk, p-p38 and c-Jun in *wild type* and *Bid*$^{-/-}$ mice before and 3, 7 and 14 days after BDL. (**C**) Analysis of p-ERK, p-Jnk, p-p38 and c-Jun in *wild type* and *lpr* mice before and 3 days after BDL (positive control (+) for p-Jnk; *wild type* mouse injected with Jo-2; positive control (+) for p- p38: *Fah*$^{-/-}$ mouse injected with homogentisic acid).

5.6 Figure D and E

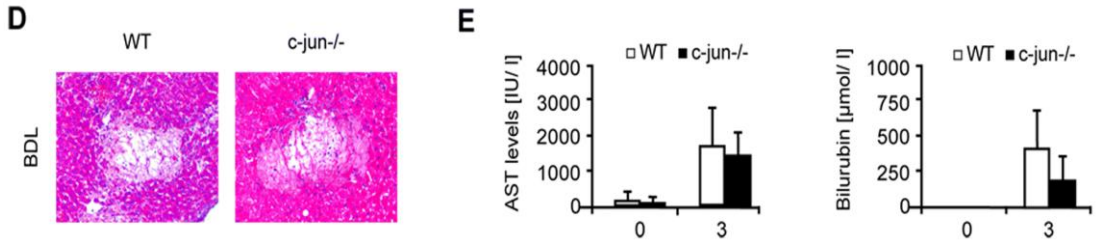


Figure 5.6 (**D**) Representative H&E staining of control and *c-Jun*$^{-/-}$ mice three days following BDL. (**E**) Serum AST and bilirubin levels were determined before and 3 days after BDL.

6.0 Role of Bid in liver injury following CCl4:

6.1. Loss of Bid attenuates liver damage following acute dose of CCl4 injection:

Following CCl4 injection, a significant increase of serum enzyme levels such as ALT and AST, as markers of liver damage, was seen in *wild type* and *Bid*$^{-/-}$ mice. ALT and AST levels peaked 48 hours following CCl4 injection and then declined at later time points. In comparison to *wild type* mice, *Bid*$^{-/-}$ mice showed significant lower levels of AST and ALT at all time points analyzed. H& E staining revealed profuse liver injury following CCL4 injection, which was less pronounced in *Bid*$^{-/-}$ mice in comparison to *wild type* mice in agreement with the biochemical data.

Figure 6.1 A and B

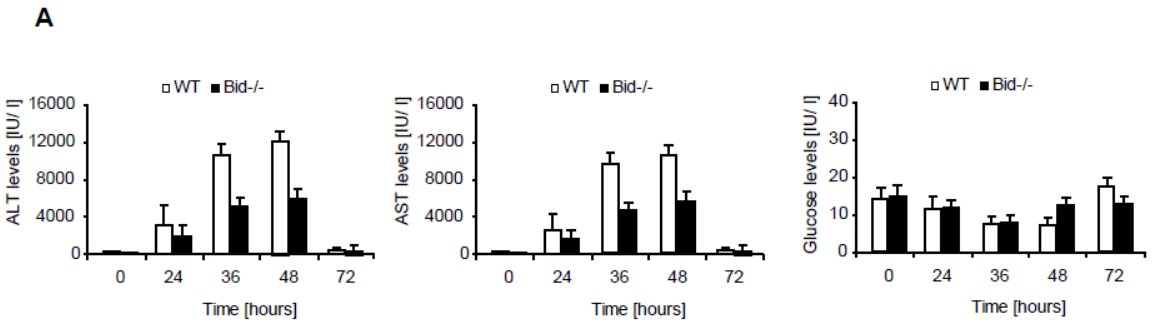
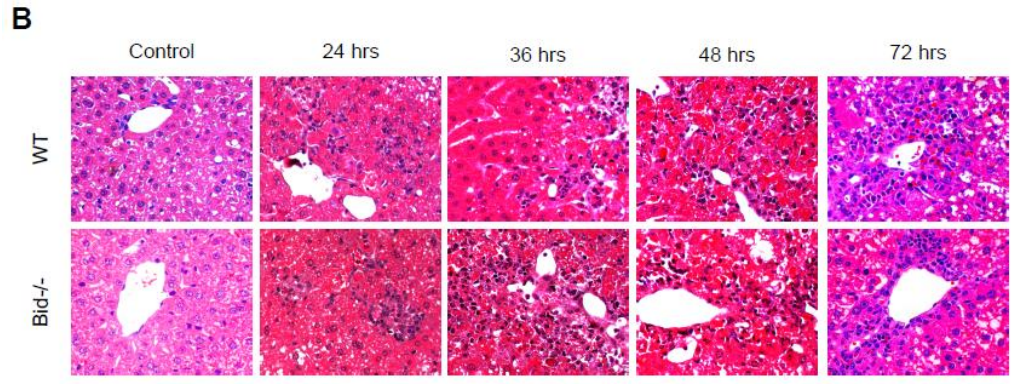


Figure 6.1 **(A)** *Wild type* and *Bid*$^{-/-}$ mice were treated with CCl4 and serum values were measured after 24, 36, 48 and 72 hours after treatment.



(B) H&E staining revealed severe damage and inflammation after CCl4 injection.

6.2 Loss of Bid doesn't affect apoptosis following acute dose of CCl4 injection:

To further assess the degree of liver injury, TUNEL staining was performed. 36 and 48 hours after CCl4 injection, intense labelling of cells was detectable in the central regions of

wild type and *Bid*$^{-/-}$ livers. In comparison to *wild type* mice, the number of positive cells was reduced in livers of *Bid*$^{-/-}$mice (Figure 6.2A). Because the TUNEL assay cannot discriminate between apoptosis and apo necrosis/ oncosis in the liver (Grasl-Kraupp et al., 1995)(Grasl-Kraupp et al. 1995), immunohistochemistry for activated (cleaved) caspase-3 was performed (Figure 6.2A).

6.2 Figure A

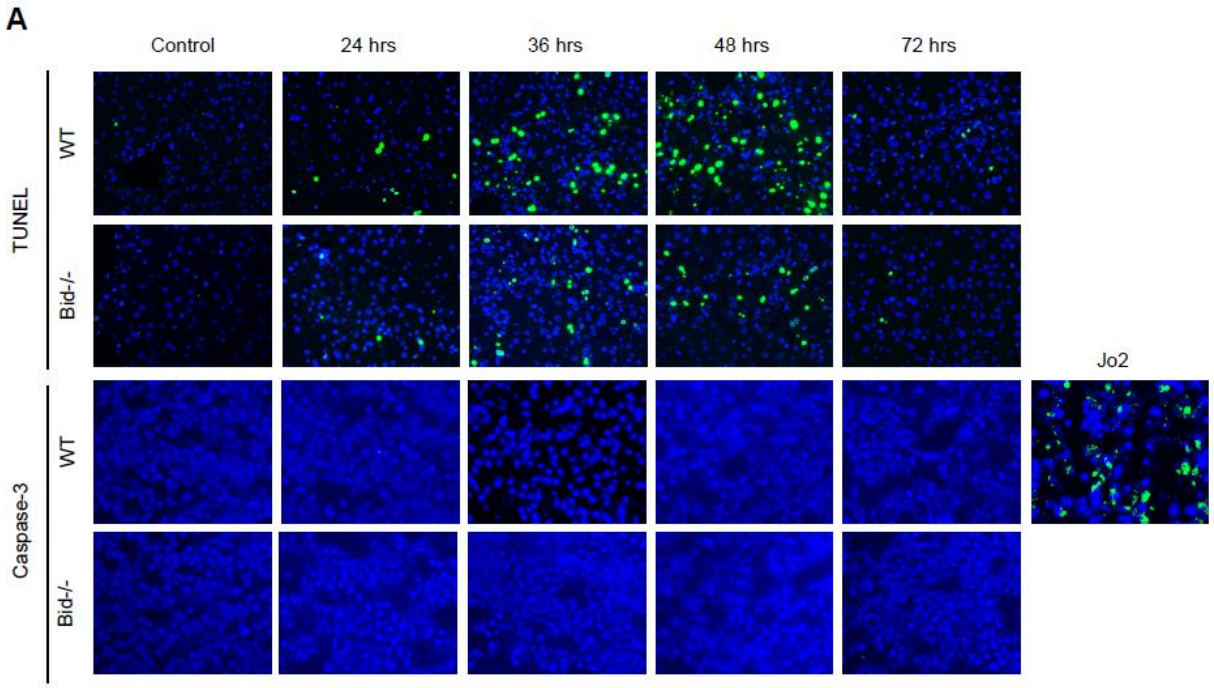
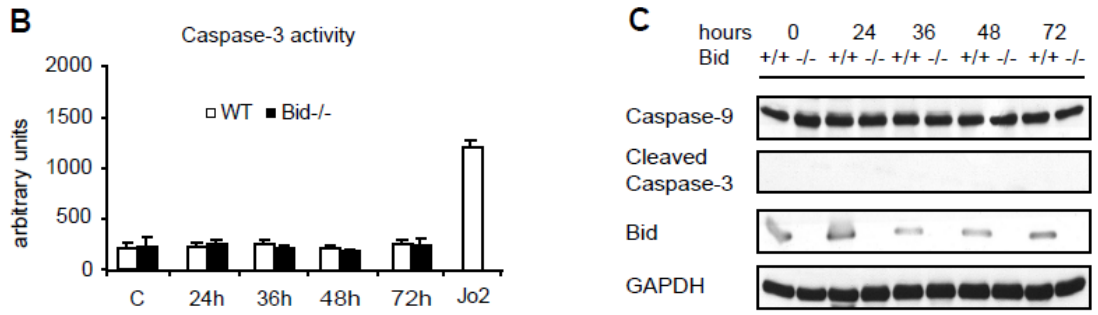


Figure 6.2 **(A)** TUNEL and Cleaved caspase-3 immunohistochemistry shown both in *Wild type* and *Bid*$^{-/-}$ mice at different time points after a single injection of CCl4.

The vast majority of TUNEL positive cells in *wild type* and *Bid*$^{-/-}$livers were negative for cleaved caspase-3, indicating that the cells were necrotic and not apoptotic. Protein lysates from livers of CCl4 treated mice were subsequently used for measurement of caspase-3 activity using caspACE Assay kit. Jo-2 treated mice were used as positive controls. Caspase-3 activity was only detected in Jo-2 treated mice but not in the CCl4 treated liver samples in agreement with the cleaved caspase-3 immunohistochemistry. Protein lysates form CCl4 treated livers were also used to detect additional markers of apoptosis such as cleavage of Bid and caspase-9 and -3 by Western blot. Bid protein levels were slightly reduced in mice challenged with CCl4, but no cleavage of caspase-9 and caspase-3 was detectable. GAPDH was used as protein loading control.



(B) caspase-3 activity was measured in the liver samples of CCl4 treated mice using CaspACE assay kit after 24, 36, 48 and 72 hours time points. **(C)** Protein lysates from CCl4 treated mice were analyzed for apoptosis markers such as cleaved caspase-9 and -3, and Bid.

6.3 Loss of Bid in proliferation following acute CCl4 injection:

We next determined whether the more severe liver damage seen in *Bid*$^{-/-}$ mice 48 h after injury was caused by a delayed onset of hepatocyte proliferation/ liver regeneration. To address this question, hepatocyte proliferation was measured by BrdU-labeling. Figure 6.3A shows BrdU and Ki 67 positive cells at different time points after CCl4 challenge in *wild type* and *Bid*$^{-/-}$ mice. BrdU and Ki 67 positive cells were quantified as shown in Figure 6.3B. 24 h after CCl4 injection, only few proliferating cells were seen in livers of *wild type* and *Bid*$^{-/-}$ mice.

6.3 Figure A

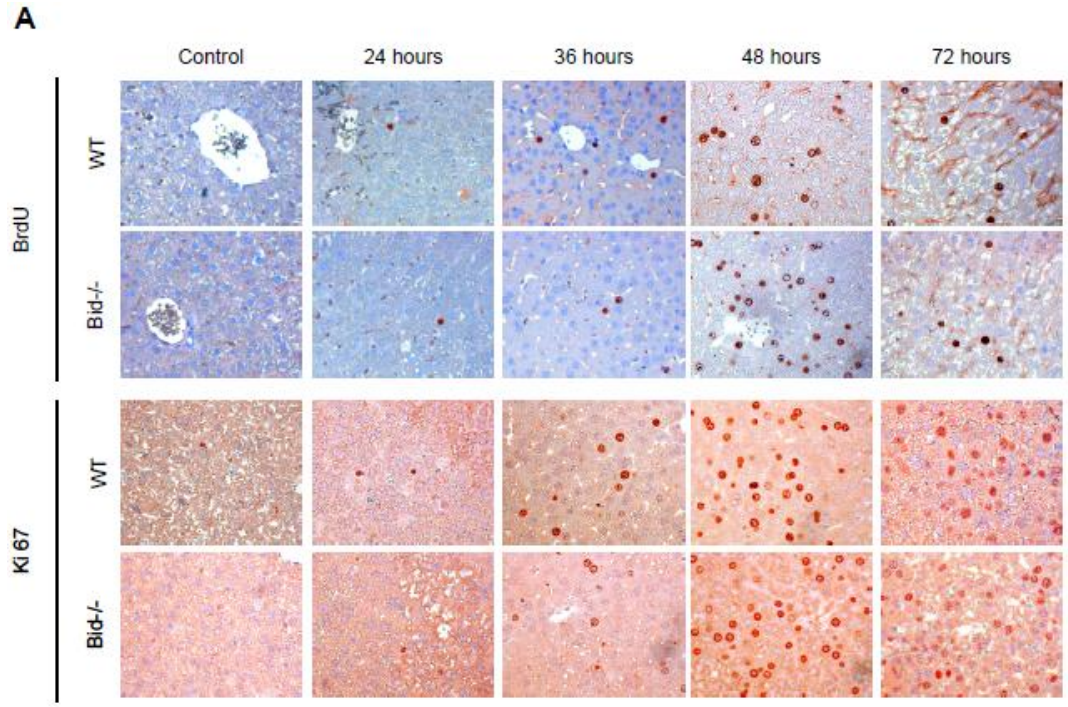


Figure 6.3 **(A)** BrdU was injected intra peritoneally 2 hours before harvesting the mice. Multiple proliferating hepatocytes were seen by BrdU and Ki 67 staining after challenge with CCl4.

After 36 h, more hepatocyte proliferation was detectable in livers of both genotypes, but the extent of proliferation was more pronounced in *wild type* mice (not significantly). At the 48 h (peak of proliferation) and the 72 h time point following CCl4 injection, the number of BrdU and Ki 67 positive cells was (not significantly) higher in *Bid*$^{-/-}$ mice compared to *wild type* mice. Protein lysate of CCl4 challenged mice were used for the detection of several key cell cycle regulatory proteins by western blot.

6.3 Figure B

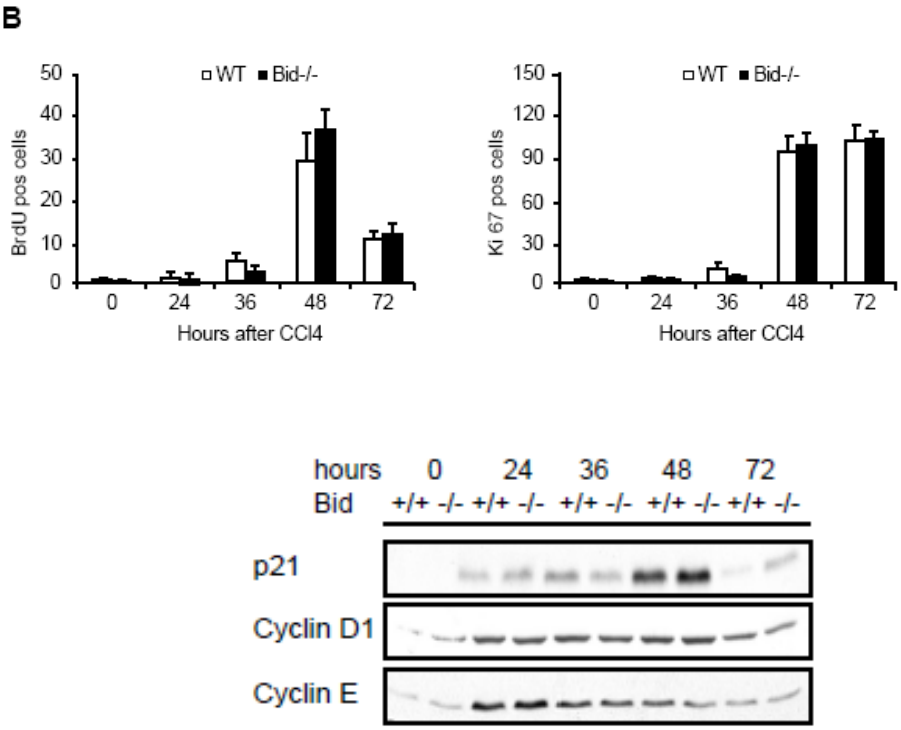


Figure 6.3 **(B)** Quantification of BrdU and Ki 67 stainings (counted at Original magnification X100). Western blots were performed for cell cycle regulating proteins p21, Cyclin D1 and Cyclin E.

Overall however, protein levels of p21, Cyclin D1 and E were similar in *wild type* and *Bid*$^{-/-}$ mice. Taken these results together, strongly suggest that the lack of Bid has no impact in liver repair after CCl4 challenge.

6.4 Loss of Bid attenuates the immunological response in *Bid*$^{-/-}$ mice following acute CCl4 damage:

We next determined whether loss of Bid affects the inflammatory response after acute CCl4 mediated liver injury. For this purpose, liver tissue was analyzed at different time points after CCl4 injection for inflammatory cells. Immunostaining with an antibody against the neutrophill marker CD11b revealed significant neutrophill infiltrates in mice of both genotypes 24 h after CCl4 challenge (Figure 6.4A), which increases subsequently in later time points (36 and 48 h after CCl4).Overall, CD11b staining was less pronounced in *Bid*$^{-/-}$ mice compare to *wild type* mice. The number of macrophages in the injured liver was determined by CD68 immunohistochemistry. Infiltration of these cells peaked 36 h and 48 h after CCl4 injection in mice of both genotypes. Western blots were performed with protein lysates for stress signaling pathway related protein (c-Jun, p-JNK, p-ERK, p-STAT3, SOCS3) and for endoplasmic reticulum (ER) and oxidative stress related proteins (CHOP, HO-1).

6.4 Figure A and B

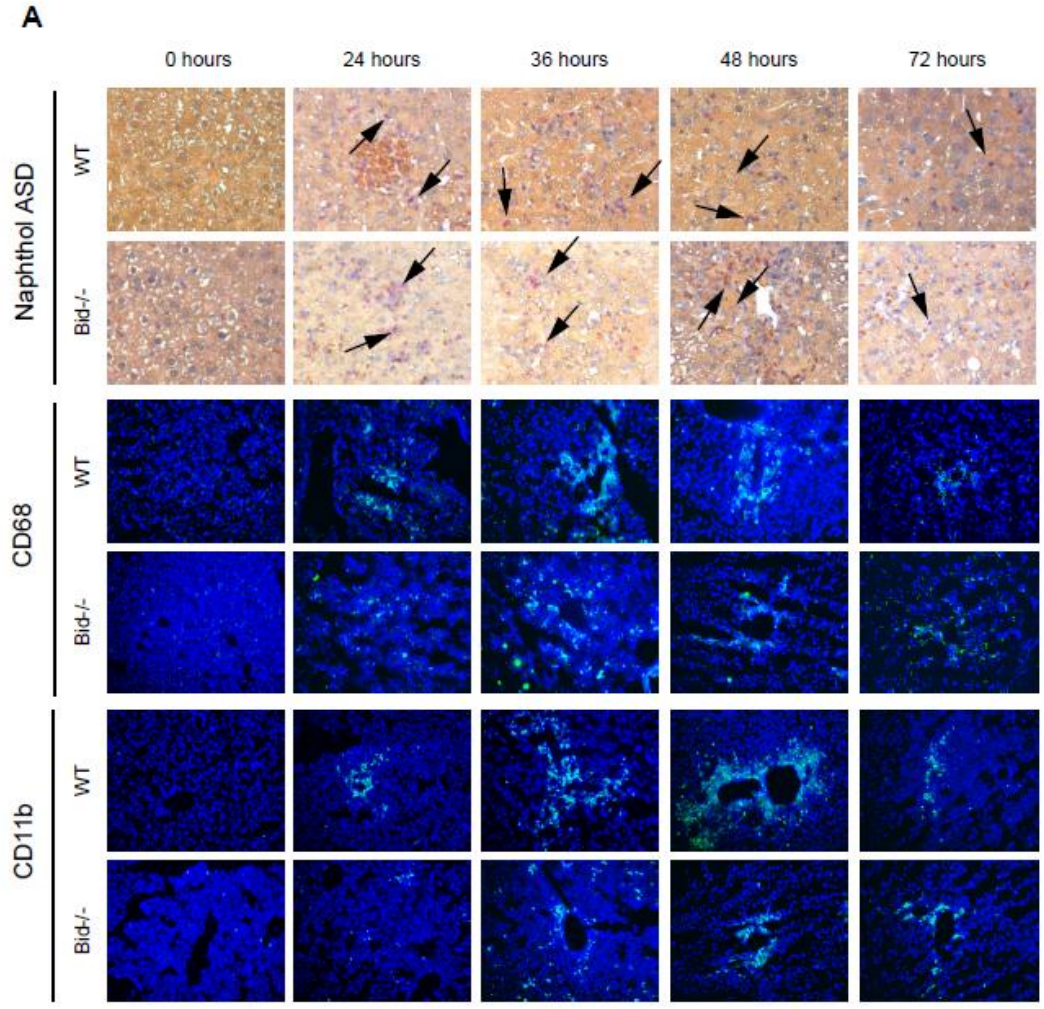


Figure 6.4 (**A**) Liver slides of control and CCl4 mice were analyzed for CD68 and CD11b expression by immunohistochemistry (Original magnification X200). Infiltrating neutrophills were detected by Naphthol ASD staining.

Protein levels of c-Jun and phosphorylation of STAT3 was slightly increased in *Bid*$^{-/-}$ mice in comparison to *wild type* mice (Figure 6.4C). In contrast, phosphorylation of ERK was initially slightly higher in *wild type* mice in comparison to *Bid*$^{-/-}$ mice, but at later time points, phosphorylation of ERK was higher in *Bid*$^{-/-}$ mice. CYP2E1 protein expression was down-regulated in all CCl4 treated mice.

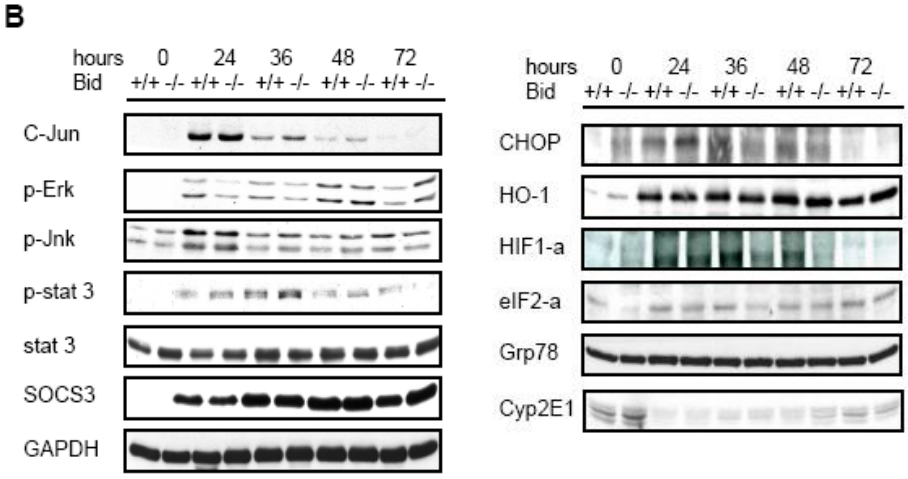


Figure 6.4 **(B)** Western blot analysis of certain stress kinase pathway proteins.

6.5 Loss of Bid accelerates liver injury following chronic liver damage:

To analyze the effect of long-term CCl4-induced liver injury**,** mice were treated twice weekly for 6 weeks with CCl4 and harvested 2 days after the last injection (12th injection).

6.5 Figure A and B

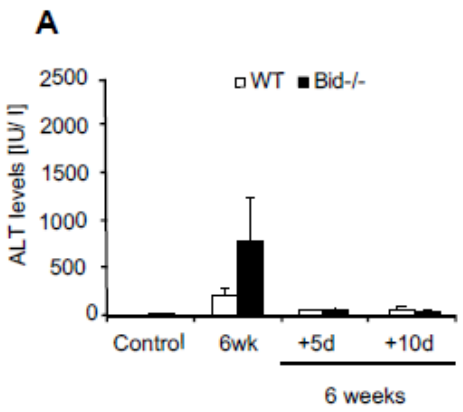
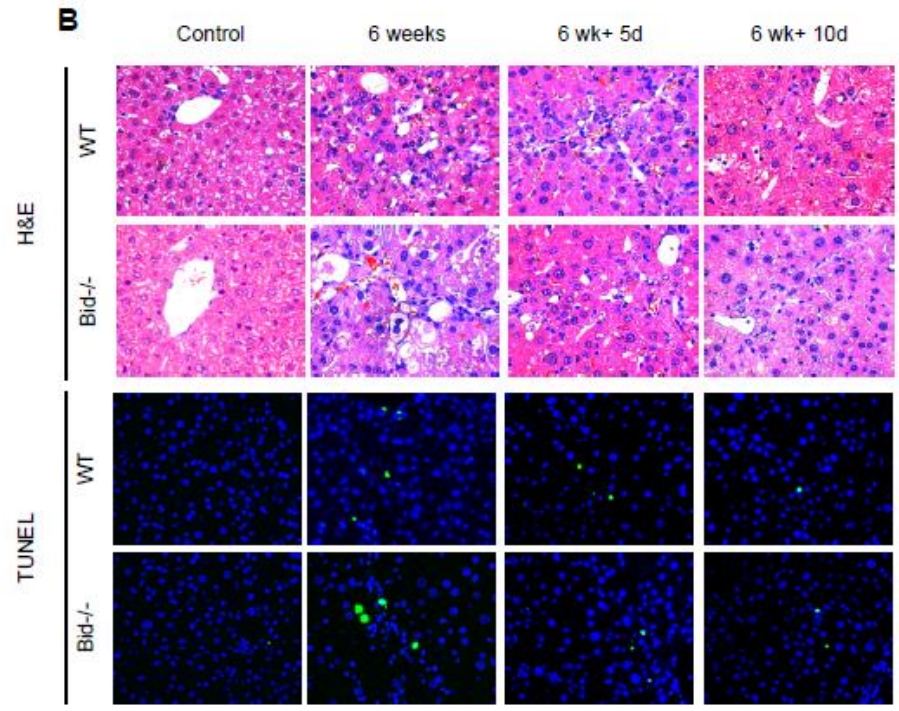


***Figure 6.5* (A)** *Wild type* and *Bid*$^{-/-}$ mice were treated with CCl4 for 6 weeks. Serum ALT levels were measured at different time points after treatment.



(B) H&E staining of liver slides revealed severe damage and inflammation after 6 weeks CCl4 treatment. Representative images are given for the different time points. TUNEL staining revealed many positive cells after 6 weeks and 6w+5d time points, (representative stainings are given at Original magnification X200).

To analyze the effect on the resolution of liver fibrosis, mice were harvested 5 days (6w+5d) and 10 days (6w+10d) after of the last injection. After 6 weeks of CCl4 treatment, levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were massive increased in *wild type* and *Bid*$^{-/-}$ mice. Surprisingly, aminotransferase activity was signify-cantly higher in *Bid*$^{-/-}$ mice in comparison to *wild type* mice at all time points analyzed (Fig. 6.5A). Furthermore, pathological analysis of liver slides with H&E staining revealed more severe damage in *Bid*$^{-/-}$ mice (Fig. 6.5B). TUNEL staining revealed multiple TUNEL positive cells in liver of mice treated for 6 weeks with CCl4, which subsequently rapidly declined (Fig.6.5B).

6.6 Loss of Bid does not affect liver fibrosis following long-term CCl4 treatment:

6.6 Figure A

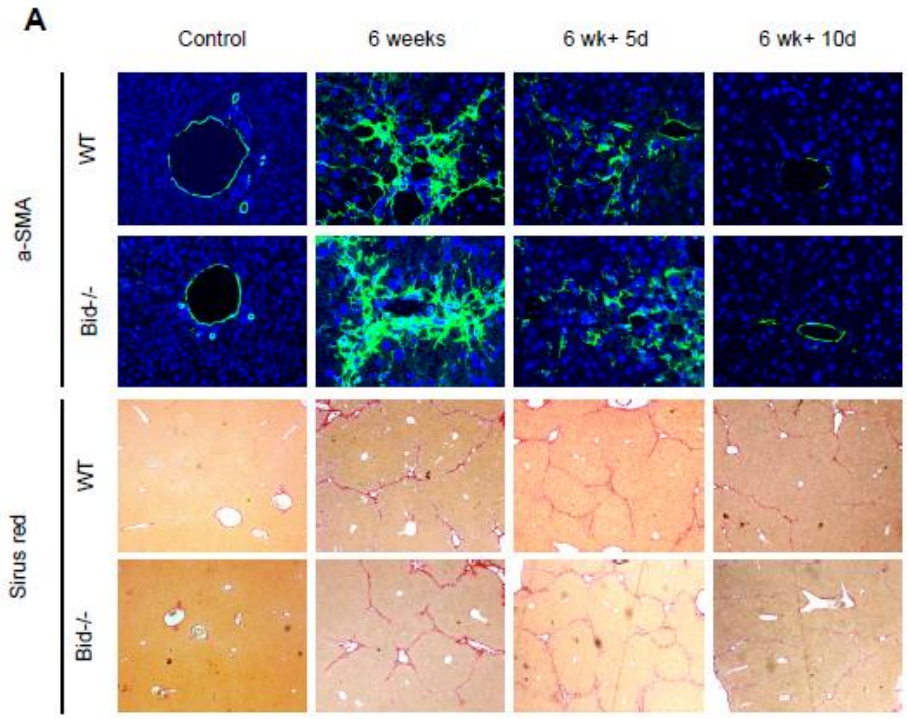


Figure 6.6: **(A)** Liver fibrosis was evaluated by a-SMA and Sirius red staining 6 weeks, 6w+5d and 6w+10d after CCl4 (Original magnification, X200).

Next liver slides were analyzed for markers of fibrosis. Alpha-sma imunohisto-chemistry and Sirius red staining showed no difference between *wild type* and *Bid*$^{-/-}$ mice

6.7 Loss of Bid in proliferation after chronic dosage of CCl4:

6.7 Figure A and B

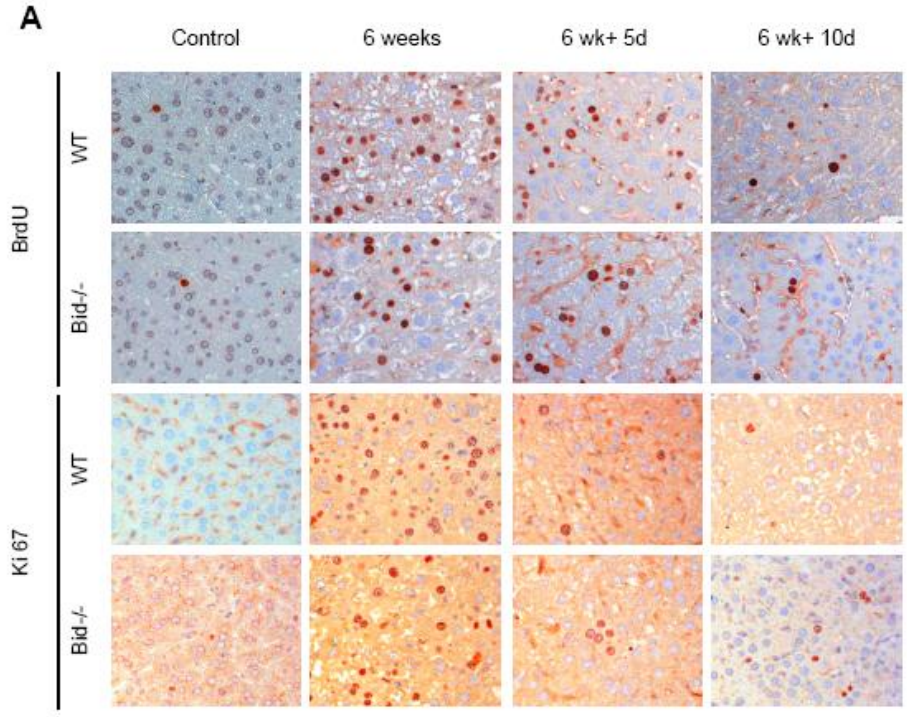
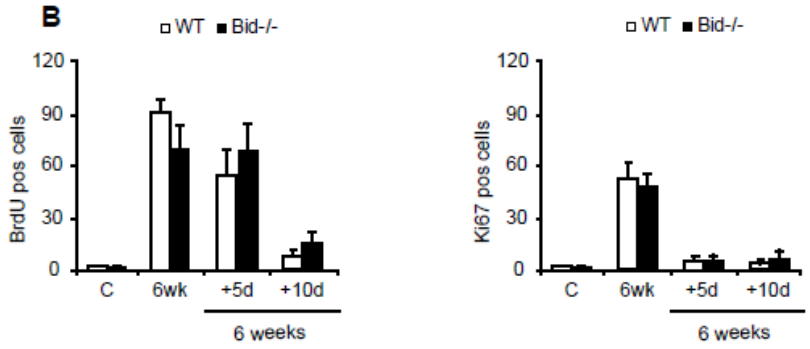


Figure 6.3 **(A)** Mice were fed with BrdU in the drinking water 4 days before harvest. BrdU immunohistochemistry and Ki 67 staining was carried out at different time points following CCl4 challenge.

To investigate how and whether loss of Bid affect hepatocyte proliferation in mice challenged with CCl4 for 6 weeks, BrdU analysis of liver slides was performed. Multiple BrdU- and Ki 67-positive nuclei were seen in mice challenged with CCl4 as shown in Fig. 6.7A. The number of positive cells subsequently declined in both groups (Fig. 6.7A). Quantification of the BrdU and Ki 67 staining did not reveal a significant difference between both genotypes (Fig. 6.7B).



(B) Quantification of BrdU and Ki 67 stainings (Original magnification X100).

7.0 Discussion:

7.1 Bid and cholestatic liver injury (bile duct ligation)

Liver injury and inflammation are common complications of cholestatic liver diseases, which can culminate in liver fibrosis and failure. Previously, it has been shown that liver injury in obstructive cholestasis is at least partially mediated by death receptors. Additionally, several *in vitro* and *in vivo* studies provided evidence that Bid is an important downstream effector of death receptors (Takikawa et al., 2001). *In vivo*, Bid antisense oligonucleotides reduce liver injury following bile duct ligation. Furthermore, toxic bile acids such DCA induce cleavage of Bid and translocation of the activated fragment of Bid to the mitochondria even in the absence of Fas *in vitro* (Higuchi et al., 2001). The primary objective of our study was therefore to analyze whether Bid affects liver injury and overall survival of mice following BDL using a genetic model.

In agreement with previous studies, multiple TUNEL positive cells were observed in livers of BDL mice. However, *Bid*$^{-/-}$ mice displayed the same pattern of TUNEL positive hepatocytes as *wild type* controls following BDL. Furthermore, AST levels, as biochemical

marker for oncosis, the degree of tissue necrosis, and the overall survival was similar in *wild type* and *Bid*$^{-/-}$ mice. Together, these data suggest that loss of Bid does not ameliorate BDL induced liver injury in contrast to its role in Jo-2-induced liver failure. This observation raises the question why pan-caspase inhibitors are protective in BDL induced liver injury (Canbay et al., 2004). Interestingly, one very recent study revealed that Trail/ DR-5 mediated apoptosis of cholangiocytes is an important determinant in BDL induced liver injury, which induces an inflammatory response within the liver and subsequently hepatocyte oncosis (Takeda et al., 2008). The mitochondrial pathway of apoptosis plays only a minor role in DR-5 mediated apoptosis of cholangiocytes, which might explain why loss of Bid did not affect BDL induced liver injury while caspase inhibitors are protective.

A secondary objective of our study was to determine whether Bid affects any other aspect of bile acid induced liver injury. There is increasing evidence that Bcl-2 like proteins do not only regulate apoptosis but also proliferation. Previously, it has been shown that deletion of Bid impedes hepatocyte proliferation during chemical induced hepatocarcinogenesis and following partial hepatectomy (Bai et al., 2005). Loss of Bid however did not affect hepatocyte proliferation during cholestatic liver injury. Next, the contribution of Bid to hepatic fibrogenesis was analyzed. Fas ligand induces proliferation of quiescent stellate cells (Reinehr et al., 2008); on the other hand, stellate cells are increasingly sensitive to Fas-mediated apoptosis following activation and apoptosis of activated stellate cells has been implicated in the resolution phase of hepatic fibrogenesis (Gong et al., 1998). Bid could therefore have divergent effects on hepatic fibrogenesis if it is important for Fas-mediated signaling in stellate cells. Our results however do not support a role for Bid in the activation or perturbation of stellate cell activation. Expression of markers for stellate cell activation such as α-Sma and TGF-β were similarly expressed in *wild type* and *Bid*$^{-/-}$ mice. Additionally, a similar degree of fibrosis at the protein levels was detected by Sirius red staining and hydroxyproline measurement.

In contrast to *Bid*$^{-/-}$ mice, Fas receptor-deficient *lpr* mice have significantly reduced liver injury following BDL. Furthermore, *lpr* mice display a reduced expression of chemokines/ cytokines and hepatic neutrophill infiltration following BDL (Gujral et al., 2003). Similarly, activation of Fas receptor by Jo-2 induces CXC chemokines MIP-2 and KC causing an active recruitment of neutrophills to the hepatic parenchyma (Faouzi et al., 2001). The mechanism by which Fas-R promotes inflammation is so far not completely understood. Previously it has been suggested that the Jo-2-mediated inflammatory response in the liver was independent on NFkB, but requires activation of caspase-3 and nuclear translocation of

AP-1 (Faouzi et al., 2001). Here, chemokine expression following BDL occurred without any significant caspase activation. Additionally, activation of stress kinase pathways was not significantly different between *wild type* and *lpr* mice. Interestingly, c-Jun was differently expressed in livers of *lpr* mice; however *c-Jun*$^{-/-}$ mice displayed a similar number of oncotic foci as *wild type* mice suggesting that the reduced c-Jun protein levels in *lpr* mice are a consequence and not a cause of the reduced liver injury in BDL mice. Therefore, an alternative way to explain the reduced liver injury in BDL mice could be that the entire effect is independent of the Fas-R expression on hepatocytes. There is evidence that *lpr* mice have fewer NKT cells and thus may generate fewer inflammatory mediators (Gujral et al., 2004). In support of this argument, it was shown that NKT cell depletion protects against BDL-induced liver injury (Kahraman et al., 2008). Together, our data suggest that Bid-mediated signaling does not play a role for death receptor-mediated liver injury in obstructive cholestasis. Our findings strongly argue against selecting Bid as a therapeutic target in cholestasis and should therefore have implication for the development of new treatment strategies during bile acid induced liver injury.

7.2 Bid and toxic liver injury (CCl4)

The liver is the largest organ in the body and the site where drugs and chemicals are primarily metabolized and detoxified. Toxins produced during metabolism may give rise to injury, but the healthy liver is capable of repairing itself. Previously it has been shown, that CCl4 inactivates cytochrome oxidase in mitochondria (Ikeda et al., 1998) and that mitochondria play a key in CCl4-induced liver injury (Susin et al., 1998). Furthermore, it has been suggested that CCl4 does not only causes necrosis, but also induces apoptosis in mouse livers (Shi et al., 1998), which might sustain inflammation and liver injury. However, the role of Bid and the mitochondrial apoptosis pathway for CCl4 induced liver injury is unclear. The aim of this study was therefore to analyze how loss of Bid affects the acute and chronic CCl4 induced-liver injury.

In agreement with previous studies acute CCl4 challenge of mice caused severe liver injury as shown by a massive increase of serum ALT and AST levels. Interestingly, transaminase levels were significantly lower in *Bid*$^{-/-}$ mice in comparison to *wild type* mice. In agreement with the biochemical data, histological analyses of the liver revealed less injury in *Bid*$^{-/-}$ mice in comparison to *wild type* mice after a single CCl4 challenge. To elucidate whether CCl4 caused apoptosis of hepatocytes, TUNEL and caspase-3 activation was analyzed. TUNEL staining revealed many positive cells disseminated throughout the liver of

CCl4 treated mice. Consistent with the biochemical and histological data, the number of TUNEL-positive cells was significantly lower in *Bid*$^{-/-}$ mice in comparison to WT mice. Similar to the BDL mice however, there were hardly any cleaved caspase-3-positive cells. Consistent with the immunohistochemical data, there was no activation and processing of caspase-9 and -3 evident in CCl4 treated mice suggesting that the strong TUNEL labeling reflects necrosis and not apoptosis (Ansari et al., 1993). Interestingly, the reduced liver injury in *Bid*$^{-/-}$ mice correlated with fewer infiltrating neutrophills into the liver. Together these data suggest that CCl4 treatment does not induce hepatocyte apoptosis as previously suggested, but more surprisingly that liver injury was nevertheless significantly reduced in *Bid*$^{-/-}$ mice.

CCl4 triggers massive hepatocyte proliferation to counter balance cell death (Xu et al., 2008); (Kwon et al., 2003). We therefore wondered whether loss of Bid affects liver regeneration following CCl4-induced liver injury. In agreement with previous studies, multiple proliferating hepatocytes were detectable in CCl4 challenged mice. Despite the more severe liver injury a similar number of proliferation hepatocytes was detectable in *Bid*$^{-/-}$ and WT mice suggesting that loss of Bid does not compromise liver regeneration following toxic injury. Next the role of Bid in chronic CCl4 induced liver injury was analyzed. Surprisingly biochemical and histological markers of liver injury were significantly more severe in *Bid*$^{-/-}$ mice following long-term treatment suggesting that loss of Bid might affect the adaptation of hepatocytes during chronic liver injury. Liver regeneration measured by BrdU and Ki67 staining was not compromised in *Bid*$^{-/-}$ mice as shown in mice challenged with a single dose of CCl4. Furthermore, *Bid*$^{-/-}$ mice did not develop more liver fibrosis than *wild type* mice despite the more pronounced liver damage.

In conclusion, our data indicate that loss of Bid ameliorates CCl4 induced acute liver injury even though CCl4 did not induced significant hepatocyte apoptosis. Reduced liver injury after a single CCL4 injection correlated with an attenuated inflammatory response (neutrophill infiltration) which might contribute to the observed phenotype. In contrast however, loss of Bid prevented the adaptation of hepatocytes against CCl4 causing more severe liver damage in *Bid*$^{-/-}$ mice treated with CCl4 for 6 weeks in comparison to WT mice. Surprisingly, aggravated liver injury in *Bid*$^{-/-}$ mice did not cause more liver fibrosis/ cirrhosis in long-term treated mice. Furthermore, loss of Bid did not affect the resolution of liver fibrosis. Additional studies will be necessary to further dissect the role of Bid in toxic CCl4-liver injury. Our data however indicate that Bid plays a role in the liver beyond its role in mediating hepatocyte apoptosis.

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Publications

Nalapareddy P, Schüngel S, Hong JY, Manns MP, Jaeschke H, Vogel A. Am J Pathol. **2009** Sep;175(3): PMID: 19661444; The BH3-only protein bid does not mediate death-receptor-induced liver injury in obstructive cholestasis.

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