Increased hepatic fibrosis and JNK2-dependent liver injury in mice exhibiting hepatocyte-specific deletion of cFLIP

Jörn M. Schattenberg,1* Michael Nagel,1* Yong Ook Kim,2 Tobias Kohl,1 Marcus A. Wörns,1 Tim Zimmermann,1 Arno Schad,3 Thomas Longerich,4 Detlef Schuppan,1,2 You-Wen He,5 Peter R. Galle,1 and Marcus Schuchmann1

1. Department of Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany; 2. Center for Molecular and Translational Medicine, University Medical Center of the Johannes Gutenberg University, Mainz, Germany; 3. Institute of Pathology, University Medical Center of the Johannes Gutenberg University, Mainz, Germany; 4. Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany; and 5. Department of Immunology, Duke University Medical Center, Durham, North Carolina

Submitted 14 December 2011; accepted in final form 25 May 2012

Schattenberg JM, Nagel M, Kim YO, Kohl T, Wörns MA, Zimmermann T, Schad A, Longerich T, Schuppan D, He Y, Galle PR, Schuchmann M. Increased hepatic fibrosis and JNK2-dependent liver injury in mice exhibiting hepatocyte-specific deletion of cFLIP. Am J Physiol Gastrointest Liver Physiol 303: G498–G506, 2012. First published June 14, 2012; doi:10.1152/ajpgi.00525.2011.—Chronic liver disease promotes hepatocellular injury involving apoptosis and triggers compensatory regeneration that leads to the activation of quiescent stellate cells in the liver. The deposition of extracellular matrix from activated myofibroblasts promotes hepatic fibrosis and the progression to cirrhosis with deleterious effects on liver physiology. The role of apoptosis signaling pathways in the development of fibrosis remains undefined. The aim of the current study was to determine the involvement of the caspase-8 homologue cellular FLICE-inhibitory protein (cFLIP) during the initiation and progression of fibrosis. Liver injury and fibrosis from carbon tetrachloride (CCl4) and thioacetamide (TAA) were examined in mice exhibiting a hepatocyte-specific deletion of cFLIP (flip−/−). Acute liver injury from CCl4 and TAA were enhanced in flip−/− mice. This was accompanied by increased activation of caspase-3 and -9, pronounced phosphorylation of JNK, and decreased phosphorylation of Erk. Deletion of the Junn NH2-terminal kinase 2 (JNK2) in flip−/− mice protected from injury. Hepatic fibrosis was increased at baseline in flip−/− mice, and progression of fibrosis from TAA was accelerated compared with the wild type. In conclusion, deletion of cFLIP in hepatocytes leads to increased fibrosis and accelerated fibrosis progression. This is accompanied by increased injury involving the activation of caspases and JNK2. Thus predisposition to liver injury involving increased hepatocellular apoptosis is a critical mediator of accelerated fibrogenesis, and prevention of liver injury will be a most important measure for patients with chronic liver disease.

Chronic liver disease is an important cause of overall mortality due to the complications that arise from end stage liver disease and cirrhosis. In recent years, advances in understanding the pathophysiology and mechanisms of hepatic fibrogenesis have been made (22). Fibrosis develops as a consequence of chronic liver injury from various causes and represents a deleterious effect during hepatic tissue regeneration. Among other mechanisms the release of humoral factors from dying hepatocytes promotes the transactivation of quiescent progenitor cells to activated myofibroblasts. This cell population is characterized by the expression of the α-smooth muscle isoform of actin (αSMA) and consists of contractile fibrogenic cells among which stellate cells have been most intensively studied. Excess deposition of extracellular matrix proteins including the fibrillar collagens type I and III leads to hepatic tissue remodeling with changes of the physiological liver architecture and vasculature (15). These histological changes promote the development of parenchymal nodules surrounded by scar tissue and the formation of vascular shunts that underlie the clinical phenotype in patients with end-stage liver disease (9).

Based on the relevance of hepatocyte cell death during fibrosis progression, understanding the underlying mechanisms of liver injury will help to identify potential therapeutic targets for antifibrotic therapies (5). In nonalcoholic steatohepatitis (NASH), serum markers that predict the degree of hepatocellular apoptosis correlate with the histological severity of the disease, indicating that hepatocellular injury is directly related to the degree of fibrosis (12). In chronic viral hepatitis, the degree of inflammation was shown to predict the relative risk of fibrosis progression and prevention of hepatocyte injury slows and reverses fibrosis progression (7, 18).

We (21) have previously shown that deletion of the caspase-8 homologue cFLIP sensitizes hepatocytes towards apoptosis from tumor necrosis factor-α (TNF) and CD95 signaling involving enhanced activation of the mitogen-activated protein kinase (MAPK) cJun NH2-terminal kinase (JNK). The role of caspase-8 in hepatocytes-induced apoptosis was recently confirmed in other studies that demonstrated that deletion of caspase-8 protected from induction of apoptosis (2, 16). To address the relevance of hepatocyte-specific deletion of cFLIP during fibrogenesis, we assessed fibrosis at baseline and following treatment with carbon tetrachloride (CCl4) or thioacetamide (TAA) in mice with hepatocyte-specific deletion of cFLIP or deletion of JNK2 (jnk2−/−) and mice exhibiting a double-knockout for cFLIP and JNK2 (DKO). We observed increased fibrosis at baseline in mice with cFLIP deficiency and increased progression of fibrosis from CCl4 and TAA in parallel to increased liver injury. Liver injury was dependent on the activation of caspases and JNK2. These findings support the hypothesis that the degree of liver injury is a key determinant for the pace of fibrosis progression. Interestingly, the absolute amounts of fibrosis were similar if liver injury persisted longer and occurred independent of JNK2.

* J. M. Schattenberg and M. Nagel contributed equally to this work.

Address for reprint requests and other correspondence: J. M. Schattenberg, Bldg. 601, Rm. 1.24, J. Dept. of Medicine, Univ. Medical Center of the Johannes Gutenberg Univ. Mainz, Langenbeckstraße 1, 55131 Mainz, Germany (e-mail: schatten@uni-mainz.de).

0193-1857/12 Copyright © 2012 the American Physiological Society http://www.ajpgi.org
MATERIAL AND METHODS

Animal model. Conditional cFLIP knockout mice were generated as previously described (21). JNK2 knockout mice (jnk2<sup>-/-</sup>) were purchased from the Jackson Laboratory (Sacramento, CA) and crossed with homozygous floxed cFLIP mice and albumin-cre carrying mice to generate jnk2<sup>-/-</sup> / albumin-cre::c-FLIP<sup>lox/lox</sup> DKO mice. All animals were bred at the animal facility of the University Medical Center of the Johannes Gutenberg University according to the criteria outlined by the Guide for the Care and Use of Laboratory Animals and approved by the Committee for Experimental Animal Research.

Acute liver injury was induced in male mice aged 8–12 wk by intraperitoneal injection with CCl<sub>4</sub> (2 µl/g body wt; Sigma Chemicals, Heidelberg, Germany) dissolved in mineral oil. For chronic injury, CCl<sub>4</sub> was used at 0.63 µl/g and injected twice a week. When indicated animals were pretreated with zVAD or DMSO (both Biomol, Hamburg, Germany) for 60 min before treatment. TAA was given over 4 wk three times a week by oral gavage in escalating dose, using 100 mg/kg at the first dose, 200 mg/kg at 2nd and 3rd, and 300 mg/kg at week 3 and 4. Fibrosis was quantitated by hydroxyproline measurement as previously described (17). Serum was obtained by retroorbital bleeding or cardiac puncture from anesthetized mice following overnight fasting.

Immunohistochemistry. Immunohistochemistry was performed on paraffin embedded tissue using antibodies specifically detecting activated caspase-3 (Cell Signaling, NS. 9661) or activated caspase-9 (Abcam no. 52298). Visualization was performed using anti-rabbit secondary antibodies (Vector Labs, Burlingame, CA). Results are expressed in percent as number of positive cells per high-power field in 10 representative fields ± SE.

Histological analysis. For histological analysis, liver samples were fixed for 36 h in 4% paraformaldehyde and stained using hematoxylin and eosin or Sirius red. Grading of fibrosis was performed using the Metavir scoring system (0 = no fibrosis, 1 = portal fibrosis without septa, 2 = portal fibrosis with few septa, 3 = numerous septa without cirrhosis, and 4 = cirrhosis) by experienced liver histopathologists in a blinded fashion. Results are expressed as means ± SE. For in situ detection of apoptotic cells, terminal deoxynucleotidyl transferase-mediated labeling of nick-end DNA (TUNEL) staining was performed according to the manufacturer’s instructions (Roche). TUNEL-positive cells were quantitated by scoring of 10 independent high-power fields and are expressed as TUNEL-positive hepatocytes divided by all hepatocytes per high-power field × 100.

Western blot. Western blotting was performed as previously described (19). Primary antibodies included the following: actin (Sigma), phospho-JNK, JNK, phospho-Erk, Erk, cytochrome c, and cytochrome oxidase IV (all Cell Signaling Technology, Danvers, MA). Membranes were exposed to goat anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (Sigma).

Preparation of cytosolic and mitochondrial protein from liver tissue. Tissue pieces were rinsed in cold PBS and homogenized using a dounce homogenizer (Fisher) in buffer solution containing 20 mM HEPES (pH), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose. The homogenate was spun at 3,000 rpm (750 g), and the supernatant was collected. After a repeated spin, the supernatant was centrifuged at 11,000 rpm (10,000 g) for 15 min. The resulting pellet was dissolved in buffer solution and stored as mitochondrial fraction. The supernatant was saved as cytosolic fraction.

RT-PCR analysis. Total RNA was isolated using TRI reagent (Sigma), and cDNA was generated using the Bio-Rad iScript cDNA synthesis kit according to the manufacturer’s specifications. Duplicate PCR amplifications were carried out in a Light Cycler LC480 (Roche) using QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden). mRNA was quantitated by relative quantification with external standards (hypoxanthine-guanine phosphoribosyl transferase-specific primers). Primers for RT-PCR were purchased from Qiagen.

RESULTS

Hepatocyte-specific deletion of cFLIP augments CCl<sub>4</sub>-induced liver injury involving caspase activation. The caspase-8 homologue cFLIP is a critical regulator of tissue homeostasis, and we (21) have previously shown that deletion of cFLIP in hepatocytes results in increased apoptotic liver injury involving caspase and MAPK activation from CD95 and TNF signaling. To determine the role of cFLIP in fibrogenesis, we choose to examine CCl<sub>4</sub>- and TAA-induced liver injury and fibrosis in mice exhibiting a liver-specific knockout of cFLIP. Acute liver injury from CCl<sub>4</sub> was significantly enhanced in flip<sup>−/−</sup> mice compared with wild-type mice. Serum ALT was increased 1.8-3.9-, 2.3-fold at 12, 24, and 48 h, respectively (Fig. 1, A and B). Histologically the degree of liver injury was significantly augmented in flip<sup>−/−</sup> mice with confluent areas of cell death concentrated around the central vein (Fig. 1C). To determine the degree of apoptosis immunohistochemistry for activated caspases-3 and -9, TUNEL assay was performed. Following treatment with CCl<sub>4</sub>, the maximum number of TUNEL-positive hepatocytes was observed at 24 h. Flip<sup>−/−</sup> mice exhibited a significantly higher number of TUNEL-positive hepatocytes most prominent around the central vein. This was accompanied by a high degree of activated caspase-3 and 9 in flip<sup>−/−</sup> mice (Fig. 1D). Quantification of hepatocytes positive for activated caspase-3 (Fig. 1E) and caspase-9 (Fig. 1F) revealed a 15.5-respiration 21.1-fold increase in caspase activation in the flip<sup>−/−</sup> mice at 24 h.

Acute injury in mice with cFLIP-deficient hepatocytes is dependent on the activation of caspases and JNK2. To determine the mechanisms of increased injury in flip<sup>−/−</sup> mice, the involvement of MAPK and caspases was examined. In mice exhibiting hepatocyte-specific deletion of cFLIP, liver injury was significantly blunted from pretreatment with the pancaspase inhibitor zVAD at 24 and 48 h (Fig. 2A: flip<sup>−/−</sup> CCl<sub>4</sub> ± zVAD at 48 h: 9,482 ± 1,156 vs. 4,349 ± 906 U/l; P < 0.05; n = 10) and reached levels that were equal to wild-type mice without zVAD pretreatment at 24 h and lower at 48 h. Wild-type mice exhibited a trend towards reduced liver injury at later time points that did not reach significance up until 48 h. On histological examination, zVAD pretreatment decreased the size of areas with cell death in flip<sup>−/−</sup> mice from CCl<sub>4</sub> at 24 h (Fig. 2B).

Prolonged activation of the MAPK JNK involving phosphorylation of the p38 and p46 isoforms was previously shown to promote liver injury from oxidative stress and to occur during CD95- and TNF-mediated liver injury (21, 24). We examined the degree of JNK phosphorylation in wild-type and flip<sup>−/−</sup> mice following treatment with CCl<sub>4</sub> at 24 h (Fig. 2). We also (21) have previously shown that deletion of cFLIP in the extracellular regulated kinase (Erk) is another membrane-expressed in percent as number of positive cells per high-power field in 10 representative fields ± SE. For in situ detection of apoptotic cells, terminal deoxynucleotidyl transferase-mediated labeling of nick-end DNA (TUNEL) staining was performed according to the manufacturer’s instructions (Roche). TUNEL-positive cells were quantitated by scoring of 10 independent high-power fields and are expressed as TUNEL-positive hepatocytes divided by all hepatocytes per high-power field × 100.

Western blot. Western blotting was performed as previously described (19). Primary antibodies included the following: actin (Sigma), phospho-JNK, JNK, phospho-Erk, Erk, cytochrome c, and cytochrome oxidase IV (all Cell Signaling Technology, Danvers, MA). Membranes were exposed to goat anti-rabbit or anti-mouse secondary antibodies conjugated with horseradish peroxidase (Sigma).

Preparation of cytosolic and mitochondrial protein from liver tissue. Tissue pieces were rinsed in cold PBS and homogenized using a dounce homogenizer (Fisher) in buffer solution containing 20 mM HEPES (pH), 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose. The homogenate was spun at 3,000 rpm (750 g), and the supernatant was collected. After a repeated spin, the supernatant was centrifuged at 11,000 rpm (10,000 g) for 15 min. The resulting pellet was dissolved in buffer solution and stored as mitochondrial fraction. The supernatant was saved as cytosolic fraction.

RT-PCR analysis. Total RNA was isolated using TRI reagent (Sigma), and cDNA was generated using the Bio-Rad iScript cDNA synthesis kit according to the manufacturer’s specifications. Duplicate PCR amplifications were carried out in a Light Cycler LC480 (Roche) using QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden). mRNA was quantitated by relative quantification with external standards (hypoxanthine-guanine phosphoribosyl transferase-specific primers). Primers for RT-PCR were purchased from Qiagen.

Statistical analysis and densitometry. All numerical results are expressed as means ± SE and represent data from a minimum of three independent experiments. Calculations were made with Sigma Plot 2000 (SPSS Science, Chicago, IL). Densitometry was performed using ImageJ software 1.44p (National Institutes of Health).
ylation of p44/42 in response to CCl₄ treatment that did not occur in flip⁻/⁻ mice. Levels of total Erk served as a control and were unchanged between both groups (Fig. 2C). To determine a mechanistic involvement of JNK in CCl₄-induced liver injury, we generated double-knockout mice carrying a deletion of JNK2 (jnk2⁻/⁻) and hepatocyte-specific deletion of cFLIP (DKO). These DKO mice exhibited a significant reduction of liver injury from CCL₄, and serum ALT was comparable to wild-type mice at 24 and 48 h (Fig. 2D). Levels of total JNK and phosphorylation of the p54 and p46 isoform were significantly decreased in jnk2⁻/⁻ and DKO mice (Fig. 2E).

Apoptosis in hepatocytes is dependent on the release of mitochondrial factors that are required for the activation of caspase-3 (10). To examine the degree of activation of the

Fig. 1. Deletion of cellular FLICE-inhibitory protein (cFLIP) in hepatocytes promotes acute liver injury and caspase activation from carbon tetrachloride (CCl₄). Wild-type and flip⁻/⁻ mice were injected with CCl₄ and examined at the indicated time points. Controls were treated with oil only. Alanine aminotransferase (ALT; A) and aspartate aminotransferase (B; AST) were determined and results are expressed as means ± SE from 3 independent experiments with each 6–8 animals for each genotype (*P < 0.05). At 48 h of the indicated treatment, hematoxylin and eosin-stained liver tissue (C) and immunohistochemistry (D) for activated caspase-3, caspase-9 and terminal deoxynucleotidyl transferase-mediated labeling of nick-end DNA (TUNEL) staining was performed. Relative activation of caspase-3 (E) and -9 (F) was determined by quantification of positive cells relative to all cells derived from 10 high-power fields. Results are expressed as means ± SE (*P < 0.05).
mitochondrial cell death signaling pathway in CCl4-treated mice, we assessed the release of cytochrome c following treatment (Fig. 2F). Cytochrome c was only detectable in the mitochondrial fraction of hepatocytes and increased approxi-
mitochondrial from the cytosolic fraction. Thus CCl4-induced acute liver injury in flip^-/- mice is dependent on the activation of caspases and JNK2 and leads to the release of proapoptotic cytochrome c from mitochondria.

**Increased hepatic fibrosis in flip^-/- mice and the expression of regulators of cellular survival during the acute phase of liver injury from CCl4.** Liver injury from CCl4 leads to the activation quiescent stellate cells. The activated phenotype exhibits increased expression of aSMA and promotes the deposition of extracellular matrix. This effect was shown to be reversible upon withdrawal of CCl4 indicating that it is a dynamic process (17). To assess hepatic fibrosis, we performed Sirius red staining, quantitated fibrosis using the Metavir scoring system, and measured hydroxyproline content in flip^-/- and wild-type mice untreated and following acute CCl4 exposure. At the age of 12 wk, untreated and oil-only-treated flip^-/- mice exhibited increased portal fibrosis as determined by histological analysis and significantly increased hepatic hydroxyproline content compared with wild-type mice (Fig. 3, A–C). To assess stellate cell activation from acute CCl4 treatment, the expression of profibrogenic markers was determined. Compared with wild-type mice, we observed a trend towards increased levels of collagen I in flip^-/-, while levels of tissue inhibitors of metalloproteinases and aSMA were increased from treatment but did not differ significantly between genotypes (Fig. 3, D-F). Despite the differences in cellular injury, no differences with regards to the expression levels of the proapoptotic factors of the Bcl-2 family Bid, Bax, and Bad were detected (Fig. 3, G-I). Thus hepatocyte-specific deletion of cFLIP in mice results in an spontaneous phenotype with increased hepatic fibrosis at the age of 12 wk.

**Liver injury and fibrosis during chronic application of CCl4 and TAA in flip^-/- mice.** Next, we sought to determine whether chronic application of CCl4 augmented the spontaneous phe-
notype and resulted in increased amounts of fibrosis. To induce fibrosis, oil or CCl₄ were injected repetitively and the severity of liver injury was assessed by histology and serum ALT at 4, 8, and 12 wk of treatment. Chronic CCl₄ application resulted in hydropic swelling and hypoxic vacuoles in hepatocytes of both genotypes (Fig. 4A). Fibrosis was assessed by Sirius red staining and quantitated using the Metavir scoring system. CCl₄ induced a strong fibrogenic response in both wild-type and flip⁻/⁻ mice with comparable Metavir scores at 8 and 12 wk (Fig. 4B) and a trend towards higher ALT levels at week 8 and J2 (Fig. 4C). In parallel, increased amounts of TUNEL-positive hepatocytes were detectable in flip⁻/⁻ mice during chronic application of CCl₄ (Fig. 4D).

To quantitate the profibrogenic response the expression profibrogenic markers was assessed. Flip⁻/⁻ mice exhibited increased aSMA and collagen I expression was at 8 wk, while this difference was lost at 12 wk (Fig. 5, A and C). Levels of tissue inhibitors of metalloproteinases were unchanged between the two genotypes from treatment with CCl₄ (Fig. 5B). No significant differences were observed in the expression of Bcl-2 family member between the two genotypes (Fig. 5, D–F).

To validate the role of cFLIP in a second model of fibrogenesis, TAA was applied by oral gavage and liver injury and fibrosis was determined. TAA promoted the development of fibrosis in both wild-type and flip⁻/⁻ mice (Fig. 6A). Liver injury was significantly increased in mice with hepatocyte-specific deletion of cFLIP. In parallel to the findings in CCl₄-mediated injury, deletion of JNK2 in DKO mice likewise blunted liver injury from TAA although serum ALT remained higher compared with wild-type mice (Fig. 6B). Thus liver injury from TAA in flip⁻/⁻ mice is dependent on the activation of JNK2. The degree of fibrosis measured by hepatic hydroxyproline content in flip⁻/⁻ mice was significantly increased compared with wild-type mice (Fig. 6, C and D). Interestingly, the hydroxyproline content did not decrease in DKO mice indicating, that loss of cFLIP promotes JNK2-dependent liver injury, while fibrogenesis occurs independently of JNK2 function.

**DISCUSSION**

We have previously shown that the caspase-8 homologue cFLIP exerts a protective effect during acute liver injury. Hepatocyte-specific deletion enhances liver injury and the mortality following activation of the CD95/Apo1/Fas and TNF signaling pathways and augments cell death from oxidative stress (20, 21). The current study extends these findings and identifies cFLIP as central regulator of acute and chronic liver injury.

**Fig. 4.** Liver histology in wild-type and flip⁻/⁻ mice during repetitive CCl₄ treatment. A: macroscopic appearance, hematoxylin and eosin-, and Sirius red-stained liver sections were examined following the indicated treatment in wild-type and flip⁻/⁻ mice. B: fibrosis was graded by blinded histological analysis of Sirius red stained liver sections using the Metavir scoring system. Liver injury was determined by serum ALT at the indicated time (C) points and apoptosis by quantification of TUNEL-positive hepatocytes (D) in wild-type and flip⁻/⁻ mice. Results are derived from 3 independent experiments and expressed as means ± SE (*P < 0.05).
injury from CCl₄ and TAA in vivo. Liver injury in mice lacking cFLIP is increased approximately three- to fivefold in the acute phase and is accompanied by the activation of caspase-3 and -9. Inhibition of caspases using the pancaspase inhibitor zVAD lowered ALT levels in flip⁻/⁻ mice to those observed in wild-type mice. These findings are consistent with the mechanisms that have been described for CCl₄-induced cellular injury involving the generation of reactive oxygen species, lipid peroxidation, mitochondrial dysfunction, and the release of TNF (25). Interestingly, zVAD pretreatment did not completely abolish liver injury indicating that the observed toxic effects are partly independent from caspase activation. To delineate the involved signaling pathways during the augmented injury in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined. Apoptosis in hepatocytes requires the release of proapoptotic factors from the mitochondria to activate effector caspases (10). In response to CCl₄ treatment, a strong release of cytochrome c occurred in flip⁻/⁻ mice, the mitochondrial cell death pathway was examined.
which triggers a regenerative response in the liver in an attempt to replace hepatocyte cell mass, contributes to fibrogenesis by activation of stellate cells and portal myofibroblasts (13). In the context of NASH, inhibition of IKK2, which impairs activation of the hepatoprotective factor NF-κB promotes caspase-3 activation and the release of hedgehog ligand and contributes to the activation of quiescent stellate cells and fibrosis (3, 8, 14). Thus increased hepatocellular injury is likely to contribute significantly to the development of fibrosis. Current concepts imply that fibrosis is a dynamic process and cessation of liver injury can lead to the regression of fibrosis, a process that involves immunomodulatory cells in the liver (11). It was recently shown that inhibition of the crosstalk between immune cells and stellate cells, e.g., through blockade of CCL5, ameliorated liver fibrosis (4). The current study further defines the relation of hepatocellular apoptosis, MAPK activation, and hepatic fibrogenesis. We observed a spontaneous phenotype in untreated flip<sup>−/−</sup> mice with increased amounts of hepatic fibrosis despite no evidence of increased cell death or elevated serum transaminases at an age of 12 wk. Application of CCl<sub>4</sub> and TAA promoted fibrosis in the employed mouse models. Depending on the length of treatment, we observed increased fibrosis at baseline and at 4 wk, however, similar grades of fibrosis in wild-type and flip<sup>−/−</sup> mice following long treatment. These differences are likely related to the sensitivity of the employed assays and the duration of treatment. Interestingly, deletion of JNK2, which reduced liver injury significantly, did not result in a decrease of fibrogenesis in DKO mice. The results of the current study extend the previous findings on the role of cFLIP and demonstrate that mice with hepatocyte-specific deletion of cFLIP 1 ex-

Fig. 6. Injury but not fibrosis from thioacetamide (TAA) is dependent on JNK2 activation on flip<sup>−/−</sup> hepatocytes. A: macroscopic appearance of liver and spleen in wild-type and flip<sup>−/−</sup> mice treated with TAA or vehicle (water) by oral gavage for 4 wk. Liver injury determined by ALT (B) and hydroxyproline content per 100 mg (C) and respiration per total liver (D) in wild-type, flip<sup>−/−</sup>, and mice with double knockout for JNK2 and cFLIP (DKO). Results are expressed as means ± SE (*P < 0.05 for wild-type vs. flip<sup>−/−</sup>, respiration DKO).
hibit a spontaneous phenotype with increased hepatic fibrosis at an age of 12 wk; 2) exhibit increased progression of fibrosis from chemically induced fibrosis; additionally, 3) the mechanism of cellular injury is dependent on the activation of JNK2 during the acute and the chronic phase.

In summary, mice exhibiting a hepatocyte-specific deletion of cFLIP display increased hepatic fibrosis and are prone to caspase-dependent liver injury from CCl4 and TAA involving the release of proapoptotic mitochondrial factors and activation of JNK2. Subsequently the progression of early fibrosis is augmented. Despite the differences and the mechanistic involvement of JNK2, fibrogenesis was independent of JNK2. Additionally, long-term exposure to CCl4 resulted in fibrosis irrespective of the underlying genotype. This stresses the importance to develop treatment strategies that prevent early liver injury in chronic liver disease to avoid the acceleration of fibrosis and progression to cirrhosis.

ACKNOWLEDGMENTS

We thank Dr. K. Reifenberg of the animal core facility at the University Medical Center of Mainz for support. S. Klein, I. Wagner, S. Weyer, B. Bartsch, C. Braun, and C. Waldmann provided excellent technical assistance. The study contains parts of the doctoral thesis of M. Nagel to be submitted at the Faculty of Medicine at the University of Mainz.

GRANTS

M. Schattenberg and M. Schuchmann received funding from the Deutsche Forschungsgemeinschaft.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


