

Fatal Hepatitis Mediated by Tumor Necrosis Factor $TNF\alpha$ Requires Caspase-8 and Involves the BH3-Only Proteins Bid and Bim

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SUMMARY

Apoptotic death of hepatocytes, a contributor to many chronic and acute liver diseases, can be a consequence of overactivation of the immune system and is often mediated by $TNF\alpha$. Injection with lipopolysaccharide (LPS) plus the transcriptional inhibitor D-(+)-galactosamine (GalN) or mitogenic T cell activation causes fatal hepatocyte apoptosis in mice, which is mediated by $TNF\alpha$, but the effector mechanisms remain unclear. Our analysis of gene-targeted mice showed that caspase-8 is essential for hepatocyte killing in both settings. Loss of Bid, the proapoptotic BH3-only protein activated by caspase-8 and essential for Fas ligand-induced hepatocyte killing, 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. These observations identify caspase-8 and the BH3-only proteins Bid and Bim as potential therapeutic targets for treatment of inflammatory liver diseases.

INTRODUCTION

Abnormal apoptotic death of hepatocytes is thought to be a cause or contributing factor in many chronic as well as acute liver diseases (reviewed in Bradham et al., 1998; Ding and Yin, 2004). Tumor necrosis factor ($TNF\alpha$), FasL, and related members of the TNF cytokine family have been implicated in hepatocyte killing but the signaling pathways contributing to development and progression of these diseases are presently unclear. The molecular basis of hepatocyte destruction in hepatitis can be studied

with mouse models. For example, injection of low doses of bacterial lipopolysaccharide (LPS) in the presence of the liver-specific transcriptional inhibitor D-(+)-galactosamine (GalN) or treatment with the T cell mitogen concanavalin A (ConA) causes fatal hepatocyte destruction. In both settings, $TNF\alpha$ is essential for hepatocyte killing and animal mortality (Pfeffer et al., 1993; Rothe et al., 1993; independently confirmed by us for both treatments, see Figure S1 available online). Secreted $TNF\alpha$ appears to be critical in LPS+GalN-injected mice, whereas both secreted and membrane-bound $TNF\alpha$ contribute to hepatocyte destruction in ConA-injected animals (Grivnennikov et al., 2005; Nowak et al., 2000; Pfeffer et al., 1993; Rothe et al., 1993). The mechanisms by which $TNF\alpha$ kills hepatocytes are still unclear. $TNF-R1$ has an intracellular “death domain” and therefore belongs to the “death receptor” subgroup within the $TNF-R$ family, which also includes Fas (APO-1 or CD95), DR3, and the TRAIL receptors DR4 and DR5 (Ashkenazi and Dixit, 1998). Genetic and biochemical studies, using fibroblasts from gene-targeted mice, have shown that $TNF-R1$ triggers apoptosis by binding the adaptor proteins TRADD and FADD, which facilitate recruitment and activation of the aspartate-specific cysteine protease caspase-8 (Ashkenazi and Dixit, 1998). Signaling through the c-Jun N-terminal kinase (JNK) has also been implicated in $TNF\alpha$ -induced apoptosis signaling (Baud et al., 1999; Kamata et al., 2005; Wang et al., 2006), but the death effectors acting downstream of JNK in this process have not yet been identified. Caspase-8 can proteolytically activate the proapoptotic BH3-only Bcl-2 family member Bid (Li et al., 1998; Luo et al., 1998), which triggers apoptosis by activating Bax and Bak (members of the second proapoptotic subgroup within the Bcl-2 family), either directly or indirectly by binding to pro-survival Bcl-2 family members (e.g., Bcl-x_L and Mcl-1) (Zha et al., 2000). Experiments with gene-targeted mice have shown that caspase-8 and Bid are both essential for Fas-induced apoptosis in hepatocytes, although only caspase-8 but not Bid is required for killing of T lymphoid cells (Kang et al., 2004; Kaufmann et al., 2007b; Salmena et al., 2003; Yin et al., 1999).

The roles of caspase-8 and Bid in TNF α -induced apoptosis are still unclear, although Bid deficiency was shown to afford a minor degree of protection in TNF α -treated mouse embryo fibroblasts (MEFs) *in vitro* and in hepatocytes of mice injected with LPS+GalN (Chen et al., 2007; Zhao et al., 2001). Notably, most studies on TNF α -induced apoptosis have been performed with cells in culture and very little is known about the mechanisms by which overactivation of the immune system causes TNF α -mediated immunopathological tissue destruction. Our experiments with gene-targeted mice demonstrated that TNF α -mediated hepatocyte apoptosis required caspase-8 and involved the proapoptotic BH3-only proteins Bid, activated by caspase-8, and Bim, activated by JNK, respectively. These cell-death inducers and effectors can therefore be considered potential therapeutic targets for immunopathological liver disorders.

RESULTS

Caspase-8 Is Essential for LPS+GalN-Induced Hepatocyte Destruction

Experiments with gene-targeted mice demonstrated that expression of caspase-8 within hepatocytes is essential for Fas-antibody-induced hepatocyte killing and fatal hepatitis (Kang et al., 2004). It is, however, not clear whether caspase-8 is also essential for pathological killing of hepatocytes by TNF α . In fact, several studies with cultured cells have indicated that TNF α kills cells by caspase-independent, perhaps even nonapoptotic, mechanisms (reviewed in Ding and Yin, 2004). When mice lacking caspase-8 selectively in hepatocytes (mice homozygous for *Casp8* gene flanked with loxP sites expressing a Cre recombinase transgene under control of the hepatocyte-specific albumin [Alb] promoter: *Alb-Cre-Casp8^{fl/fl}*) were challenged with LPS+GalN, they showed only minor elevation of ALT and AST in the serum (Figure 1A) and retained normal liver structure (Figure 1B), and all mice survived long-term (Figure 1C). In contrast, all littermate controls succumbed to this treatment within 8–10 hr (Figure 1C), presenting at autopsy with abnormally elevated serum ALT and AST levels (Figure 1A; *Alb-Cre-Casp8^{fl/fl}* versus control mice: $p < 0.015$ for ALT, $p < 0.0015$ for AST) and extensive disruption of liver architecture (Figure 1B). Consistent with these observations, immunoblot analysis of liver extracts from LPS+GalN-treated control animals revealed processing of Bid (p22) into its active p15 form tBid as well as extensive processing of caspase-3 and caspase-7, whereas no Bid cleavage and no activation of effector caspases could be detected in *Alb-Cre-Casp8^{fl/fl}* mice (Figure S2).

Consistent with the experiments using mice lacking caspase-8 in their hepatocytes, treatment of C57BL/6 (WT) mice with the pan-caspase inhibitor Q-VD-oph resulted in significant ($p < 0.01$) protection from LPS+GalN-induced hepatitis, as assessed by serum levels of ALT and AST as well as histological examination (Figures 1D and 1E and Figure S3A). However, administration of Q-VD-oph, even at multiple dosages, afforded less protection than loss of caspase-8 (compare Figure S3B with Figure 1C), presumably because this treatment did not achieve complete blockade of this enzyme. Similar to Fas-activation (Li et al., 1998; Luo et al., 1998), injection of WT mice with LPS+GalN caused rapid processing of pro-caspase-8 to produce the active p18 fragment and cleavage of Bid (p22) into its active truncated

p15 form (tBid), as well as processing and activation of effector caspases, such as caspase-7 (p17) (Figure 1F). No processing of caspase-8, Bid, or effector caspases was seen in liver extracts from LPS+GalN-injected mice lacking TNF α (Figure S4). Collectively, these results demonstrate that upon LPS+GalN injection, activation of caspase-8 within hepatocytes is required for TNF α -mediated liver destruction and fatal hepatitis.

Bid Is a Minor Contributor to LPS+GalN-Induced Hepatocyte Apoptosis

Caspase-8-mediated activation of Bid is essential for Fas-antibody-induced liver destruction (Yin et al., 1999). We confirmed this observation (Kaufmann et al., 2007b) and found that *Bid^{-/-}* mice are also resistant to FasL (Figure S5). This is an important finding because antibodies to Fas do not always mimic the physiological ligand FasL (Huang et al., 1999).

Although Bid cleavage is readily detectable in the livers of LPS+GalN-treated animals (Figure 1F), recently published data indicate that, in contrast to Fas signaling (Kaufmann et al., 2007a; Yin et al., 1999), TNF α -TNF-R1-induced hepatocyte apoptosis may not be entirely dependent on Bid (Chen et al., 2007). For evaluating the role of Bid in the *in vivo* killing of hepatocytes by TNF α , WT and *Bid^{-/-}* mice were challenged with various doses of LPS+GalN. Upon injection of very low doses of LPS (10 ng) plus GalN, *Bid^{-/-}* mice were clearly less severely affected than WT animals. All WT mice succumbed within 6–8 hr of treatment, whereas all *Bid^{-/-}* mice remained alive and healthy at that time, presenting with nearly normal ALT and AST serum levels (Figure 2A; *Bid^{-/-}* versus WT: $p < 0.0002$ for ALT, $p < 0.0001$ for AST) and only minor liver destruction (Figure 2C). However, only three out of seven *Bid^{-/-}* mice survived this treatment for 5 days (experiment stopped thereafter); the other four became terminally ill within 24 hr (Figure 3C). Moreover, when the dose of LPS was increased to 100 or 1000 ng, not only the WT but also all *Bid^{-/-}* animals succumbed to this treatment within 12 hr, although the serum levels of ALT and AST remained significantly lower in the *Bid^{-/-}* mice compared to the WT littermates (Figure 2B; $p < 0.02$ for ALT and $p < 0.002$ for AST). Consistent with the serum levels of ALT and AST, histological examination of liver sections taken after 6 hr showed extensive hepatocyte destruction in *Bid^{-/-}* mice (albeit slightly less than in WT controls) after injection of 100 or 1000 ng LPS+GalN (Figure 2C). These results demonstrate that Bid contributes to LPS+GalN-induced hepatocyte apoptosis, but its loss can only delay but not prevent the fatal outcome of this TNF α -mediated process.

Combined Loss of Bid and Bim Potently Protects Mice from LPS+GalN-Induced Fatal Hepatitis

Not only Bid (Li et al., 1998; Luo et al., 1998) but also the related BH3-only protein Bim (encoded by the *Bcl2l1* gene, also called *Bim*) was reported to be activated by caspase-mediated proteolysis (Chen and Zhou, 2004). Therefore, and because Bim plays a major role in apoptosis in many cell types (Bouillet et al., 1999; Huang and Strasser, 2000) and binds avidly to all prosurvival Bcl-2 family members (Chen et al., 2005; Kuwana et al., 2005), we examined whether Bid and Bim cooperate in TNF α -mediated hepatocyte killing *in vivo*. Interestingly, *Bcl2l1^{-/-}* mice were less sensitive to injection with 10 ng LPS+GalN (with two of

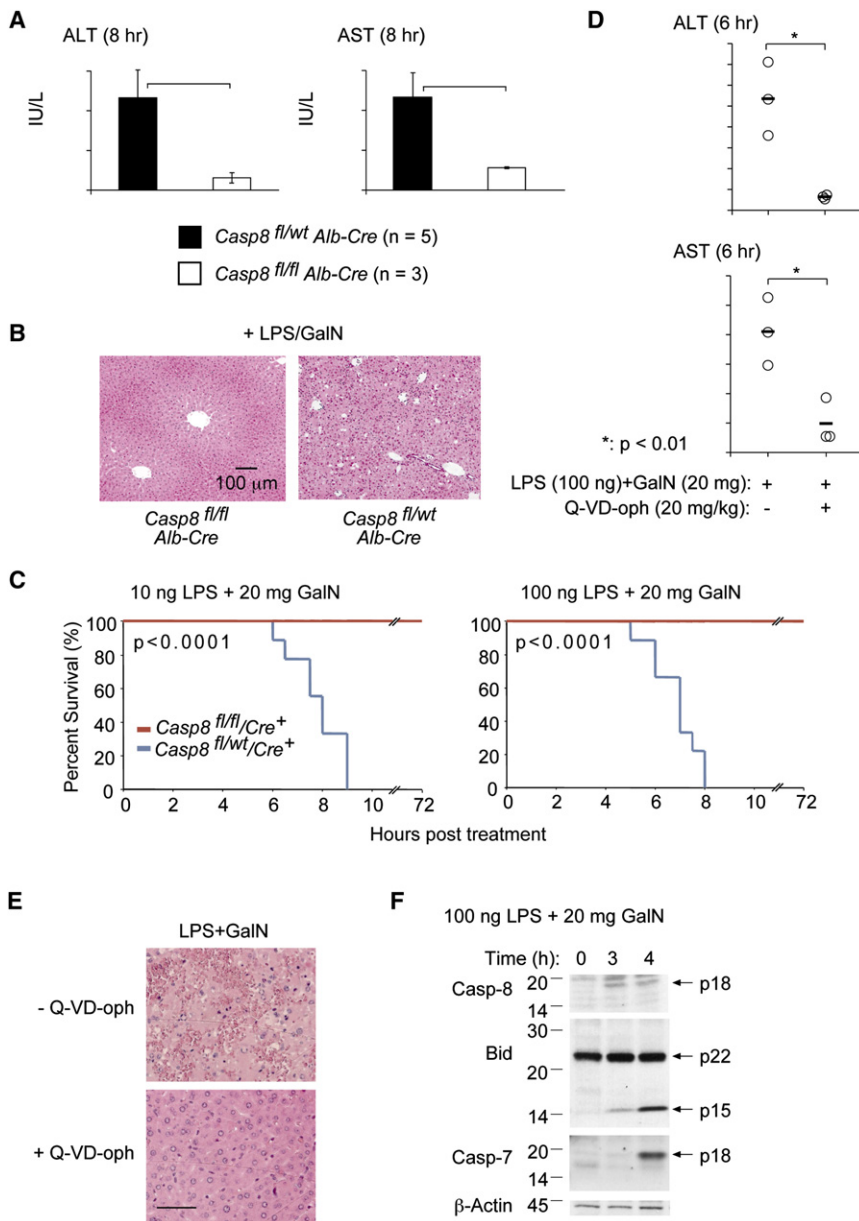


Figure 1. LPS+GalN-Induced Hepatitis Requires the Initiator Caspase, Caspase-8, Is Inhibited by a Pan-Caspase Inhibitor, and Involves Cleavage of the Proapoptotic BH3-Only Bcl-2 Family Member Bid

(A) Mice lacking caspase-8 in hepatocytes (*albumin-Cre* transgenic *Casp8^{fl/fl}*) and littermate controls (*albumin-Cre* transgenic *Casp8^{fl/wt}* mice) were injected with 100 ng LPS with the addition of 20 mg GalN. At the time when the control mice were terminally ill (~8 hr), all animals were sacrificed and serum levels of the liver transaminases ALT and AST measured. Data shown represent means \pm SD of three to five mice for each genotype.

(B) H&E-stained histological liver sections from mice treated as in (A). Pictures shown are representative of the analysis of at least three mice for each treatment and genotype.

(C) Nine mice of the indicated genotypes in each group were injected i.p. with LPS+GalN and followed over 72 hr after injection. *p* values were calculated with a time-to-event analysis with a log-rank test. No mortality was observed in mice of either genotype injected with 20 mg GalN alone (*n* = 3 per genotype).

(D) Three control WT mice and three WT mice pre-treated with 20 mg/kg of the pan-caspase inhibitor Q-VD-oph (i.p. injection 30 min prior to treatment) were injected with 100 ng LPS with the addition of 20 mg GalN. All animals were sacrificed after 6 hr and serum levels of ALT and AST measured. Each circle represents measurements from an individual mouse. Horizontal bars indicate the mean.

(E) H&E-stained histological liver sections from the same mice treated in (D). Pictures shown are representative of the analysis of three mice for each treatment.

(F) WT mice were injected with 100 ng LPS with the addition of 20 mg GalN and sacrificed after the indicated time points. Processing of caspase-8, Bid, caspase-7, and β -actin (loading control) was examined by immunoblotting.

five mice surviving long-term) than WT animals, although no protection was evident when they were challenged with 100 ng LPS+GalN (Figure 3C). Remarkably, *Bcl2l1^{-/-}Bid^{-/-}* mice were more resistant than WT, *Bcl2l1^{-/-}*, or *Bid^{-/-}* mice to both 10 and 100 ng LPS+GalN (Figure 3). At the time when all WT, *Bid^{-/-}*, and *Bcl2l1^{-/-}* mice were ill, serum ALT and AST levels of *Bcl2l1^{-/-}Bid^{-/-}* mice were significantly lower than those found in the control animals (Figure 3A; *Bcl2l1^{-/-}Bid^{-/-}* versus WT mice, *p* < 0.03 for ALT, *p* < 0.035 for AST). Histological analysis confirmed reduced liver destruction in *Bcl2l1^{-/-}Bid^{-/-}* mice compared to all control animals (Figure 3B). Furthermore, eight out of eight *Bcl2l1^{-/-}Bid^{-/-}* mice did not get sick when treated with 10 ng LPS+GalN and survived long-term (followed up to 5 days), a dose at which all WT mice succumbed within 6–8 hr and only three out of seven *Bid^{-/-}* mice and two out of five *Bcl2l1^{-/-}* mice survived longer than 24 hr (Figure 3C).

Remarkably, even at 100 ng LPS+GalN, a dose at which all WT (12/12), all *Bid^{-/-}* (12/12), and all *Bcl2l1^{-/-}* (3/3) mice succumbed within 12 hr, five out of seven *Bcl2l1^{-/-}Bid^{-/-}* mice survived long-term (followed up to 5 days), even though some went through phases of sickness during the first 24 hr of treatment (Figure 3C and Figure S6). Consistent with the finding that Bid and Bim have overlapping functions in LPS+GalN-induced hepatocyte killing, immunoblotting (Figure S7A) and fluorogenic-enzyme-activity assays (Figure S7B) showed that loss of Bid and to a lesser extent loss of Bim both diminished activation of effector caspases and that loss of both of these BH3-only proteins caused significantly (*p* < 0.01) greater reduction, particularly at later time points. Synergy between loss of Bid and loss of Bim appears to be specific to LPS+GalN-induced (TNF-mediated) hepatocyte killing because fibroblasts and T lymphocytes from *Bcl2l1^{-/-}Bid^{-/-}* mice were either normally

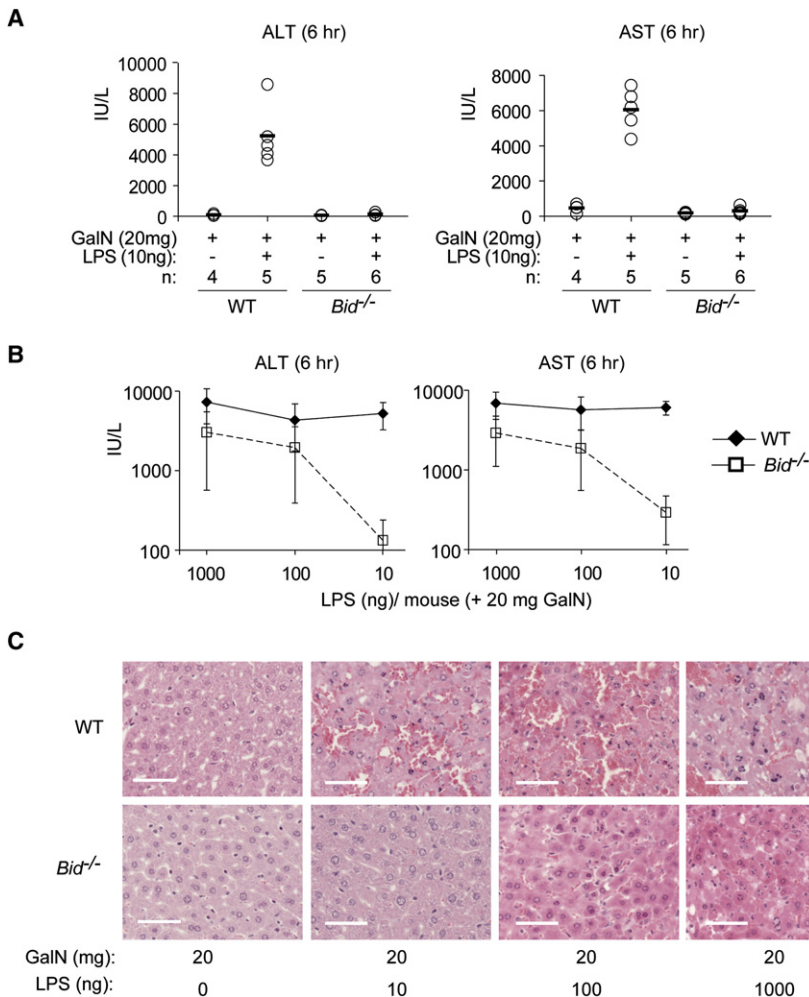


Figure 2. Bid Contributes to LPS+GalN-Induced Hepatitis

(A) Bid-deficient mice and WT controls were injected i.p. with 10 ng of LPS+GalN (20 mg per mouse) or with GalN alone as control. Mice were sacrificed after 6 hr and bled, and serum ALT and AST levels were measured. Each circle represents measurements from an individual mouse (n indicates the numbers of mice analyzed for each condition of treatments). Horizontal bars indicate the mean.

(B) Bid-deficient mice and WT controls were injected i.p. with increasing doses (10, 100, and 1000 ng) of LPS, all in the presence of 20 mg GalN, and analyzed after 6 hr as in (A). Data are presented as means ± SD.

(C) Histological examination of H&E-stained liver sections of *Bid*^{-/-} and WT mice subjected to the treatments indicated (scale bars represent 50 μm). Pictures shown are representative of the analysis of at least three mice for each treatment and genotype.

sensitive or no more resistant than *Bcl2l11*^{-/-} cells to a diverse range of apoptotic stimuli (Willis et al., 2007 and T.K., P.B., and A.S., unpublished data). These results demonstrate that the two proapoptotic BH3-only proteins Bid and Bim cooperate in TNF α -mediated hepatocyte apoptosis induced by treatment with LPS+GalN.

Mechanisms of Activation of Bid and Bim during LPS+GalN-Induced Hepatitis

Because Bid and Bim play critical overlapping roles in TNF α -induced apoptosis, we investigated how they are activated in hepatocytes from animals injected with LPS+GalN. This treatment caused rapid processing of Bid into the potentially proapoptotic truncated tBid form (Figures 1F and 4A). Loss of caspase-8 or pretreatment of WT mice with Q-VD-oph, which affords substantial protection from LPS+GalN-induced hepatitis (Figures 1D and 1E and Figure S3), decreased the production of tBid in LPS+GalN-injected mice and this correlated with a lack of pro-caspase-8 processing and a complete lack of effector-caspase activation (Figure 4A and Figure S2).

Although caspase-mediated activation of Bim_{EL} (the most abundantly expressed isoform of Bim in the liver and other tissues [O'Reilly et al., 2000]) has been reported (Chen and Zhou, 2004),

we found no evidence for Bim_{EL} cleavage in livers of LPS+GalN-injected mice (Figures 4A and 5A). JNK-mediated phosphorylation has also been implicated in the activation of Bim (Lei and Davis, 2003). Notably, a mobility shift in Bim_{EL} in liver extracts was observed soon after injection with LPS+GalN (Figures 4A and 5A and Figure S4). Treatment of these extracts with λ -phosphatase demonstrated that the modification of Bim was due to a change in phosphorylation (Figure 5B) and examination of livers from LPS+GalN-injected *Tnfa*^{-/-} mice showed that this change was TNF α dependent (Figure S4). The LPS+GalN-induced phosphorylation of Bim does not require caspase activity, given that the shift in Bim mobility on SDS-PAGE was not affected by prior administration of the pan-caspase inhibitor Q-VD-oph (Figure 4A) or loss of caspase-8 in hepatocytes (Figure 4B).

The proapoptotic activity of Bim can be regulated by its sequestration to microtubules by binding to the dynein motor complex (Puthalakath et al., 1999), and JNK-mediated phosphorylation was reported to cause release and activation of Bim in response to certain stress stimuli (Lei and Davis, 2003; Putcha et al., 2003). We therefore performed subcellular fractionation to examine whether the phosphorylation of Bim seen after LPS+GalN injection affected its sequestration to the microtubule-associated dynein motor complex. Immunoblot analysis of the microtubule-enriched pellet and soluble fractions revealed that the LPS+GalN-treatment-induced phosphorylated form of Bim was released from the microtubular dynein motor complex into the cytosol, whereas the unmodified form of Bim was preferentially detected in the dynein-motor-complex-enriched fraction (Figure 5C). Immunoblotting also revealed an increase of phosphorylated (i.e., activated) JNK in livers of LPS+GalN-injected mice (Figure 5D), and the mobility shift in Bim elicited by

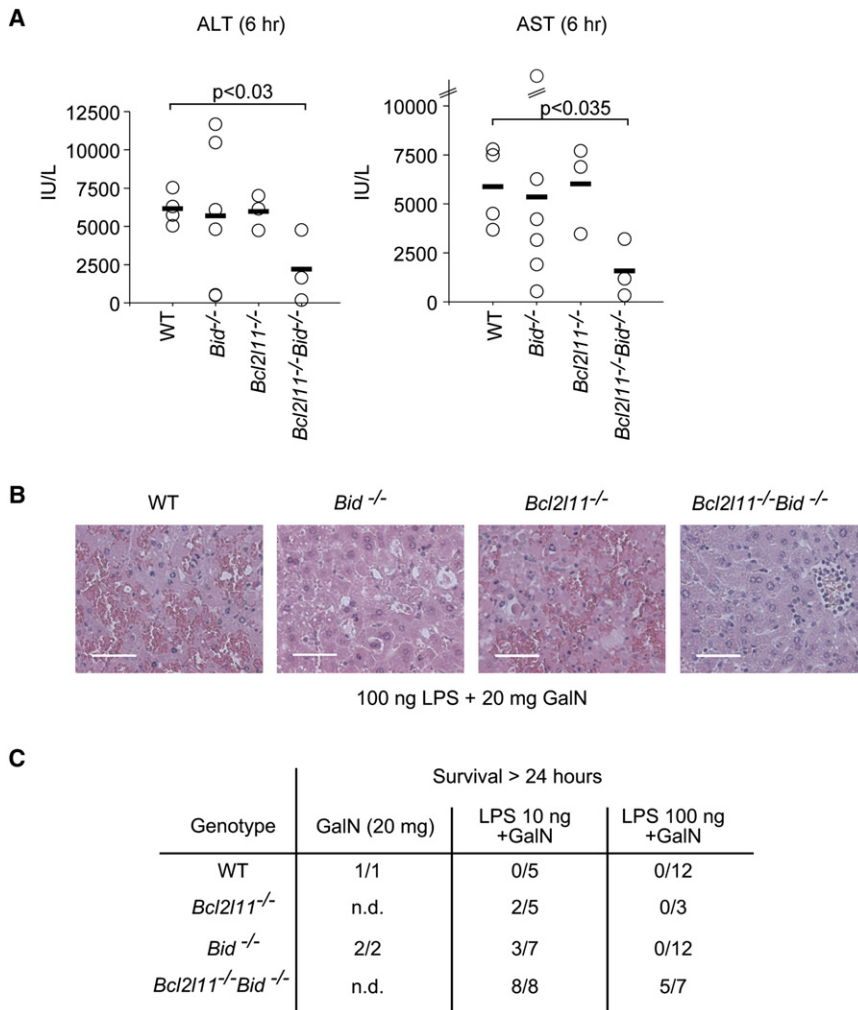


Figure 3. Loss of Bim Cooperates with Loss of Bid to Protect Mice from LPS+GalN-Induced Hepatitis

(A) Mice of the indicated genotypes were injected with 100 ng LPS plus 20 mg GalN. At the time when the WT mice were sick (6 hr), all animals were sacrificed and serum levels of ALT and AST were measured. Each circle represents measurements from an individual mouse. Horizontal bars indicate the mean.

(B) Histological examination of H&E-stained liver sections of mice of the indicated genotypes injected 6 hr earlier with 100 ng LPS plus 20 mg GalN (scale bars represent 50 μ m). Pictures shown are representative of the analysis of at least three mice for each treatment and genotype.

(C) Table summarizing the long-term (followed up to 5 days) survival of the indicated genotypes of mice treated with 10 ng or 100 ng LPS plus 20 mg GalN. Mice that did not survive this treatment all succumbed within 24 hr.

survived this treatment and showed only minor elevation of serum ALT and AST levels (Figure 7A) and retained normal liver structure (Figure 7B). In contrast, all littermate controls succumbed to this treatment within 6–8 hr, presenting at autopsy with abnormally elevated serum levels of ALT and AST (Figure 7A; caspase-8-deficient versus control mice: $p < 0.0035$ for both ALT and AST) and extensive disruption of liver architecture (Figure 7B).

In contrast to treatment with LPS+GalN, loss of Bid did not confer protection against ConA-induced hepatocyte killing. However, similarly to

this treatment was prevented by prior administration of the synthetic JNK-inhibitory D-peptide, D-JNK11 (Figure 5E). In contrast, D-JNK11 injection did not affect processing of Bid to tBid (Figure 5E), demonstrating that this inhibitor did not interfere with the LPS+GalN-induced production of TNF α or activation of caspase-8. Importantly, injection of this JNK inhibitor reduced LPS+GalN-induced hepatocyte destruction in *Bid*^{-/-} mice but not in those lacking Bim (Figures 6A–6C), consistent with the notion that JNK specifically regulates the proapoptotic activity of Bim, which cooperates with Bid in hepatocyte killing. Collectively, these results show that caspase-8-dependent activation of Bid and JNK-induced activation of Bim cooperate in TNF α -mediated hepatocyte destruction in LPS+GalN-injected mice.

Concanavalin A-Induced Hepatitis Requires Caspase-8 and Involves JNK-Mediated Activation of Bim

We next tested whether caspase-8 and the BH3-only proteins Bid and Bim are also critical for hepatocyte killing and hepatitis induced in different pathological settings. Injection of concanavalin A (ConA) into mice causes systemic activation of T lymphocytes resulting in fatal hepatitis that is mediated by TNF α (Kusters et al., 1997; Trautwein et al., 1998). When mice lacking caspase-8 selectively in hepatocytes were challenged with ConA, they

LPS+GalN injection, we observed rapid increases of phosphorylated (i.e., activated) JNK and phosphorylated Bim in livers of mice challenged with ConA (Figures 7C–7E). This modification in Bim could not be blocked by preadministration of the pan-caspase inhibitor Q-VD-oph (Figure 7F) or specific loss of caspase-8 in hepatocytes (data not shown). Although mice lacking Bim or both Bid and Bim had lower serum levels of ALT and AST compared to ConA-injected WT or *Bid*^{-/-} mice, these enzymes were still abnormally elevated (Figure 7G). Furthermore, disruption of liver architecture in *Bcl2l11*^{-/-} and *Bcl2l11*^{-/-}*Bid*^{-/-} mice after 6 hr of treatment, although less severe compared to WT or *Bid*^{-/-} mice (Figure 7H), eventually led to the death of all *Bcl2l11*^{-/-} as well as *Bcl2l11*^{-/-}*Bid*^{-/-} mice. Collectively, these results show that caspase-8 is essential and Bim a contributor in ConA-induced hepatitis, another widely used model of TNF α -mediated liver destruction.

DISCUSSION

Overstimulation of the immune system, either by pathogen-associated molecular pattern (PAMP)-mediated activation of cells of the innate immune system or antigenic stimulation of the adaptive arm, plays a major role in immunopathology of the liver as well as

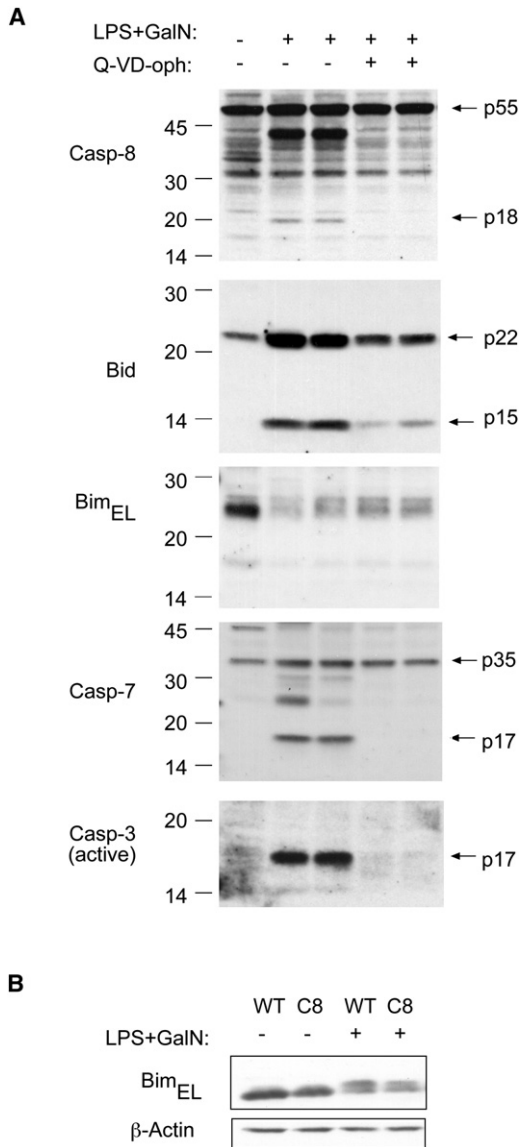


Figure 4. Treatment with LPS+GalN Causes a Posttranslational Modification of Bim that Does Not Require Caspase-8 or Other Caspases

(A) Mice (WT), with or without pretreatment with 20 mg/kg of the pan-caspase inhibitor Q-VD-oph, were injected with LPS (100 ng) plus GalN (20 mg) and sacrificed 4 hr later. Total protein extracts from the livers of these animals were probed by immunoblotting for Bim, Caspase-8, Bid, Caspase-7, and active Caspase-3.

(B) Mice lacking caspase-8 in hepatocytes (C8: *Albumin-Cre* transgenic *Casp8^{fl/fl}*) and littermate controls (*Albumin-Cre* transgenic *Casp8^{fl/wt}*) were injected with 100 ng LPS plus 20 mg GalN and sacrificed after 4 hr. Total protein extracts derived from the livers of these animals were probed by immunoblotting for Bim or β-actin (loading control).

certain other tissues. This damage is mediated to a large extent through the proapoptotic activity of TNF α (Bradham et al., 1998; Ding and Yin, 2004). The mechanisms by which TNF α kills cells under these conditions remain unclear. Using the widely employed LPS+GalN as well as the ConA injection models of fatal hepatitis, we defined an essential role for caspase-8 and impor-

tant contributory roles for the two proapoptotic BH3-only Bcl-2 family members Bid and Bim in TNF α -induced tissue destruction. When higher levels of LPS (1000 ng) were administered or with ConA injection, BH3-only proteins were no longer limiting for fatal hepatitis. In contrast, in another model of “death-receptor”-induced fatal hepatitis, mediated by Fas activation, Bid is essential for hepatocyte apoptosis (Kaufmann et al., 2007b; Yin et al., 1999). What could be the reasons for these differences? We speculate that differences in the requirement for Bid, Bim, and possibly additional BH3-only proteins for hepatocyte destruction induced by different death-receptor signals may arise from differences in caspase-8 activity. A higher extent of caspase-8 activity would be expected to cause more direct effector-caspase activation and may also increase apoptosis signaling through direct cleavage of certain vital targets, thereby rendering this pathway to hepatocyte apoptosis less dependent on Bid and other BH3-only proteins (Figure S8). It appears likely that the reduction in the caspase-8 inhibitor c-FLIP (Figure S9A), probably caused by GalN-mediated blockade of its transcriptional inducer NF- κ B (Geisler et al., 2007), contributes to the finding that caspase-8 can suffice for LPS+GalN-induced (TNF α -mediated) hepatocyte killing even in the absence of Bid (and Bim). It cannot be excluded that additional differences between TNF α - and FasL-induced hepatocyte killing may arise from the ability of TNF-R1 and TNF-R2 to trigger proapoptotic pathways in addition to caspase-8 activation that are not activated by Fas (Figure S8).

Our genetic studies show that Bim and Bid can both contribute to TNF α -induced apoptosis signaling. The BH3-only proteins Bim and Bid initiate apoptosis signaling through activation of caspase-9 and consequent activation of effector caspases by activating Bax and Bak, either directly and/or indirectly by blocking the prosurvival Bcl-2 family members that keep Bax and Bak in check (Youle and Strasser, 2008). In response to TNF α , Bid is activated by caspase-8-mediated proteolysis (Li et al., 1998; Luo et al., 1998). Although caspase-mediated activation of Bim_{EL} has been reported (Chen and Zhou, 2004), we found no evidence for Bim_{EL} cleavage in livers of LPS+GalN-injected mice. Caspase-8 might theoretically contribute to Bim activation indirectly, such as by cleaving a protein that controls its sequestration to microtubules, a critical mode of Bim regulation (Puthalakath et al., 1999), but we found no evidence for this (data not shown). Bim does, however, not necessarily have to be activated by caspase-8 to contribute to TNF α -induced apoptosis signaling. Any TNF α -induced signal that activates Bim (see below) would be expected to promote activation of caspase-9 and effector caspases (either on its own or with tBid) because Bim is a potent activator of Bax and Bak (Figure S8 and Youle and Strasser, 2008).

JNK has been implicated in TNF α -induced apoptosis signaling and JNK-mediated phosphorylation has been implicated as a trigger of the proapoptotic activity of Bim by causing its release from sequestration to the microtubular dynein motor complex (Lei and Davis, 2003; Putcha et al., 2003). Remarkably, a mobility shift in Bim_{EL}, which was prevented by addition of a highly specific JNK inhibitor, and a redistribution of the posttranslationally modified Bim from microtubules to the cytosol were observed in livers from mice injected with LPS+GalN or ConA. Importantly, injection of the JNK inhibitor reduced LPS+GalN-induced hepatocyte killing in *Bid*^{-/-} mice but not in *Bcl2l1*^{-/-} mice, which

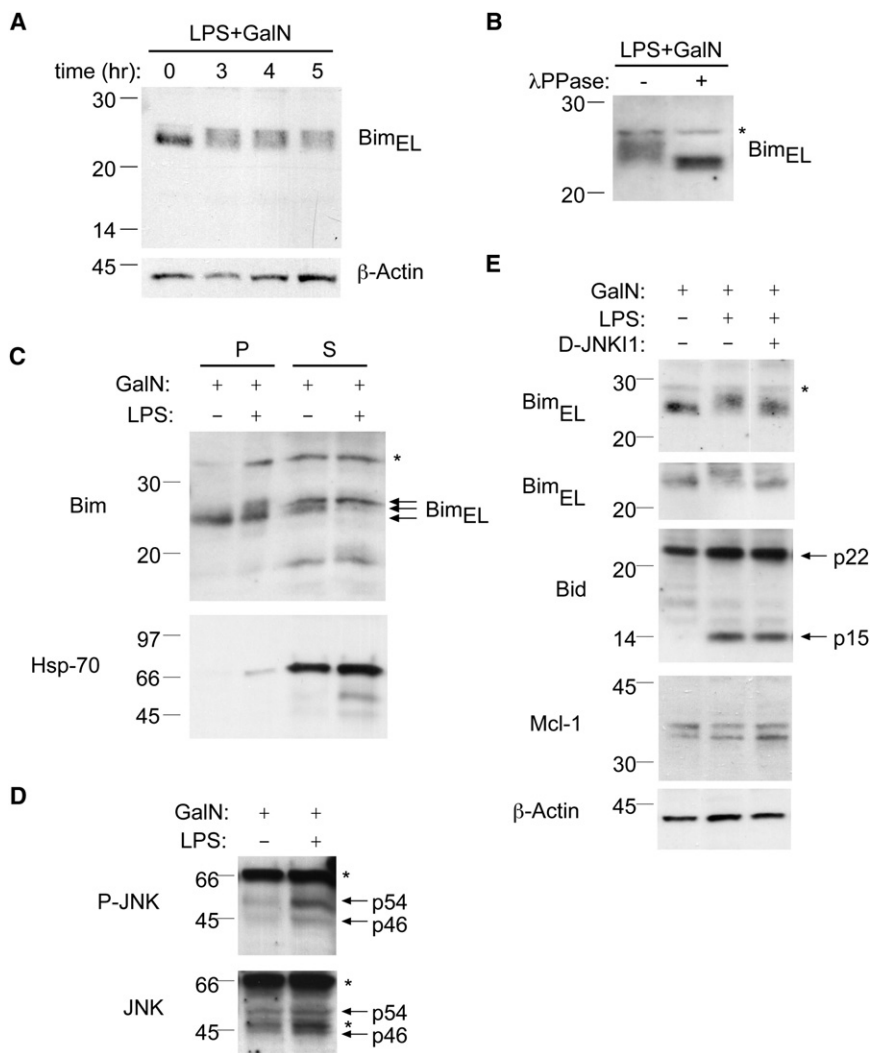


Figure 5. Bim Is Phosphorylated by JNK Kinase in the Livers of Mice Injected with LPS+GalN

(A) WT mice were injected with LPS (100 ng) plus GalN (20 mg) and sacrificed at the time points indicated. Levels and posttranslational modifications of Bim were investigated in liver-derived total protein extracts by immunoblotting. Probing with an antibody to β -actin was used as a loading control.

(B) WT mice were injected with LPS (100 ng) plus GalN (20 mg) and sacrificed after 3 hr. Total protein extracts were prepared from the liver in the absence of phosphatase inhibitors and left untreated or treated in vitro with λ -phosphatase prior to analyzing Bim modifications by immunoblotting.

(C) Mice were treated with GalN (20 mg) alone (controls) or with LPS (100 ng) plus GalN (20 mg) and livers harvested after 4 hr. Protein extracts were prepared and then subjected to subcellular fractionation into the dynein enriched pellet fraction (P), containing Bim_{EL/L} sequestered to microtubules, and a soluble fraction (S) containing free cytosolic Bim. Fractions were analyzed by immunoblotting with antibodies to Bim or HSP70 (control for the purity of the fractions).

(D) Liver extracts from WT mice treated with 100 ng LPS plus 20 mg GalN for 4 hr were probed by immunoblotting with an antibody specific for phosphorylated (activated) JNK and the membrane reprobed with an antibody to total JNK (loading control).

(E) WT mice that had either been left untreated or pretreated for 30 min with the JNK inhibitory peptide D-JNK11 (20 mg/kg, i.p.) were treated for 4 hr with either 20 mg GalN or with 100 ng LPS plus 20 mg GalN. Total liver extracts were probed by immunoblotting with antibodies to Bim (two blots from independent experiments shown), Bid, Mcl-1, and β -actin (loading control). Asterisks indicate nonspecific bands.

lack the proapoptotic effector activated by JNK. We therefore conclude that JNK contributes to TNF-R1-induced apoptosis signaling in hepatocytes of LPS+GalN-injected mice by unleashing the proapoptotic activity of Bim. ConA injection causes hepatocyte killing through both secreted and membrane-bound TNF α and therefore involves both TNF-R1 and TNF-R2, whereas LPS+GalN acts mainly through secreted TNF α , which signals only through TNF-R1 (Grivennikov et al., 2005; Nowak et al., 2000; Pfeffer et al., 1993; Rothe et al., 1993). The fact that membrane-bound TNF α can activate JNK through not only TNF-R1 but also TNF-R2 may therefore explain why Bim is more important than Bid in ConA-induced hepatocyte killing (Figure S8).

In contrast to caspase-8 deficiency, combined loss of Bid and Bim did not afford complete protection against LPS+GalN- or ConA-induced hepatocyte destruction. Some *Bcl2l1*^{-/-}*Bid*^{-/-} mice succumbed when injected with higher doses of LPS (e.g., 100 ng) plus GalN, and even some of the long-term survivors went through phases of sickness. Moreover, all *Bcl2l1*^{-/-} and *Bcl2l1*^{-/-}*Bid*^{-/-} mice injected with ConA became sick and had to be sacrificed, although they exhibited less liver damage than WT or *Bid*^{-/-} animals. This may indicate that proapoptotic

proteins in addition to Bid and Bim (perhaps other BH3-only proteins) contribute to LPS+GalN- and ConA-induced fatal hepatitis. Theoretically, TNF α might also promote apoptosis by causing a reduction in the amounts of prosurvival Bcl-2 family members, but we found no significant changes in Mcl-1 or Bcl-x_L (the most highly expressed ones in hepatocytes), in animals treated with LPS+GalN or ConA (Figures S2, S7, and S9B and not shown). As discussed above, we prefer the hypothesis that with increased caspase-8 activation (achieved by increased TNF α -signaling due to increased levels of LPS or injection of ConA), leading to increased direct activation of effector caspases and cleavage of vital proteins, hepatocyte killing may become independent of the BH3-only protein > Bax plus Bak > Apaf-1 > Caspase-9 amplification mechanism for effector-caspase activation (Figure S8). It is noteworthy that it has been speculated that at high doses of LPS, other TNF α -mediated or perhaps even TNF α -independent nonapoptotic death pathways might also be activated in hepatocytes (Kamata et al., 2005). Our experiments with mice lacking caspase-8 in hepatocytes imply, however, that such processes would have to be caspase-8 dependent.

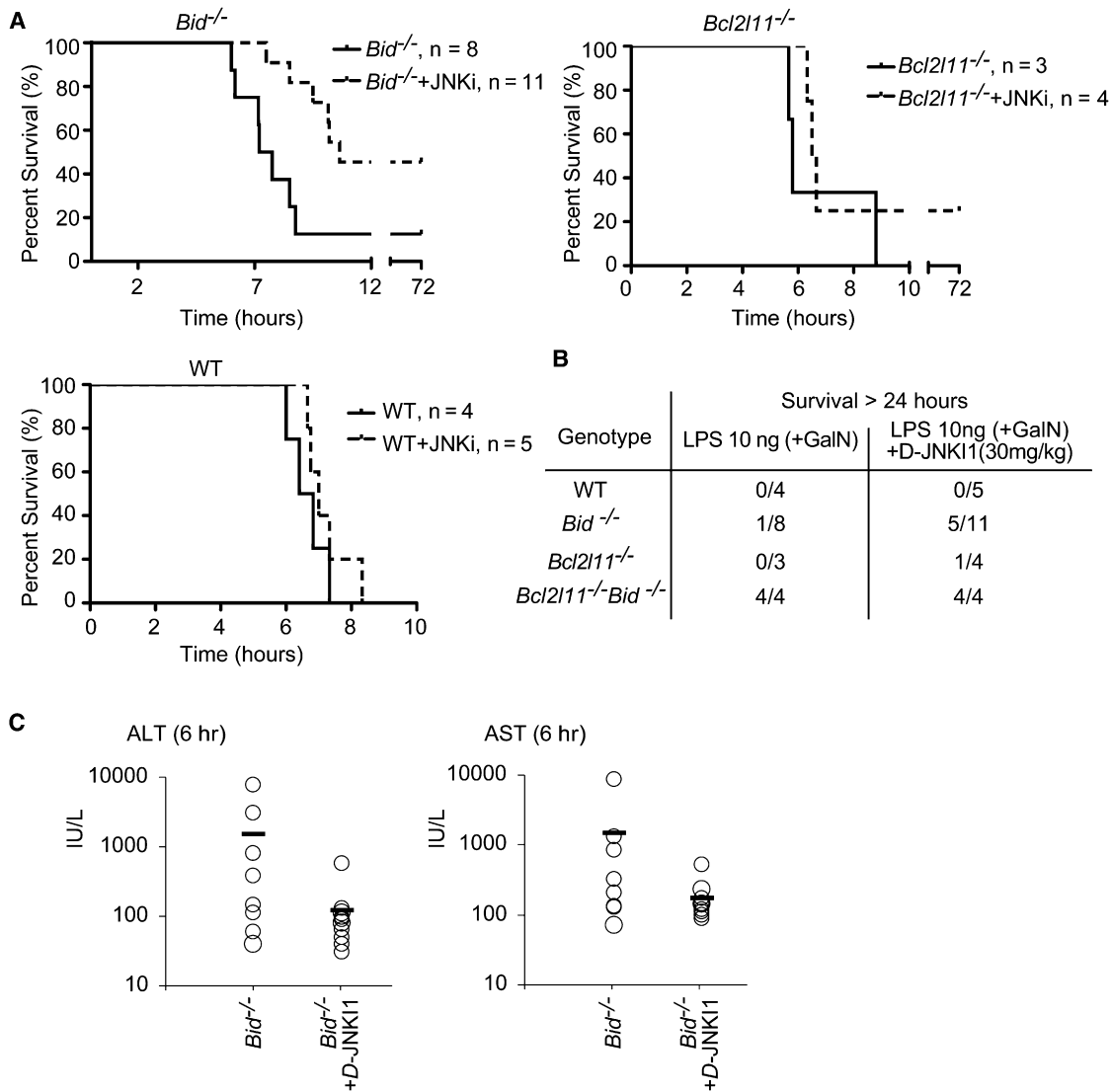


Figure 6. Inhibition of JNK Enhances Resistance of Bid-Deficient Mice to LPS+GalN-Induced Hepatocyte Destruction

(A) Mice of the genotypes indicated were injected with JNK inhibitory peptide D-JNK11 (30 mg/kg, i.p.) 30 min prior to treatment with 10 ng LPS+GalN and were then monitored for survival for up to 72 hr. Mice injected with LPS+GalN served as controls. We calculated p values with a time-to-event analysis using a log-rank test. (B) Table summarizing the long-term (followed up to 3 days) survival of mice of the indicated genotypes treated with 10 ng LPS+GalN, with or without pretreatment with D-JNK11. Mice that did not survive all succumbed to the treatment within 24 hr.

(C) Serum levels of ALT and AST in *Bid*^{-/-} mice treated with 10 ng LPS plus 20 mg GalN, with or without pretreatment with D-JNK11 (30 mg/kg), were measured after 6 hr. p values were as follows: WT versus *Bid*^{-/-}: p (ALT) = 0.09; p (AST) = 0.16. Each circle represents an individual mouse. Horizontal bars indicate the means of 8–11 mice for each genotype and are derived from three independent experiments.

In conclusion, our results show that caspase-8 is essential and the BH3-only proteins Bid and Bim are critical contributors in the pathological killing of hepatocytes that is caused by TNF α in vivo. These observations may have implications for diseases characterized by hepatocellular destruction, indicating that they might respond to treatment with enzymatic inhibitors of caspase-8 or compounds that can antagonize both Bid and Bim.

EXPERIMENTAL PROCEDURES

Mice

The generation and genotyping of Bid-deficient mice on an inbred C57BL/6 background has been described (Kaufmann et al., 2007b). *Bcl2l11*^{-/-} mice

(Bouillet et al., 1999) were originally generated on a mixed C57BL/6x129SV background by homologous recombination in 129SV-derived ES cells but have been backcrossed for more than ten generations onto the C57BL/6 background. The *Bcl2l11*^{-/-}*Bid*^{-/-} mice were generated by serially intercrossing the two parental strains. Mice lacking caspase-8 selectively in hepatocytes were generated by crossing mice with a loxP targeted *Casp8* gene, generated on a mixed C57BL/6x129SV background and backcrossed with C57BL/6 mice for three generations (Salmena et al., 2003) with transgenic mice expressing the Cre recombinase under control of the hepatocyte-specific albumin promoter (backcrossed onto C57BL/6 background for six generations). TRAIL-deficient (*Tnfrsf10*^{-/-}) mice (Cretney et al., 2002) were generated by homologous recombination in 129SV-derived ES cells and have been backcrossed for more than ten generations onto the C57BL/6 background. *TNF*^{-/-} mice (generated with C57BL/6 ES cells) (Korner et al., 1997) were obtained from H. Korner, James

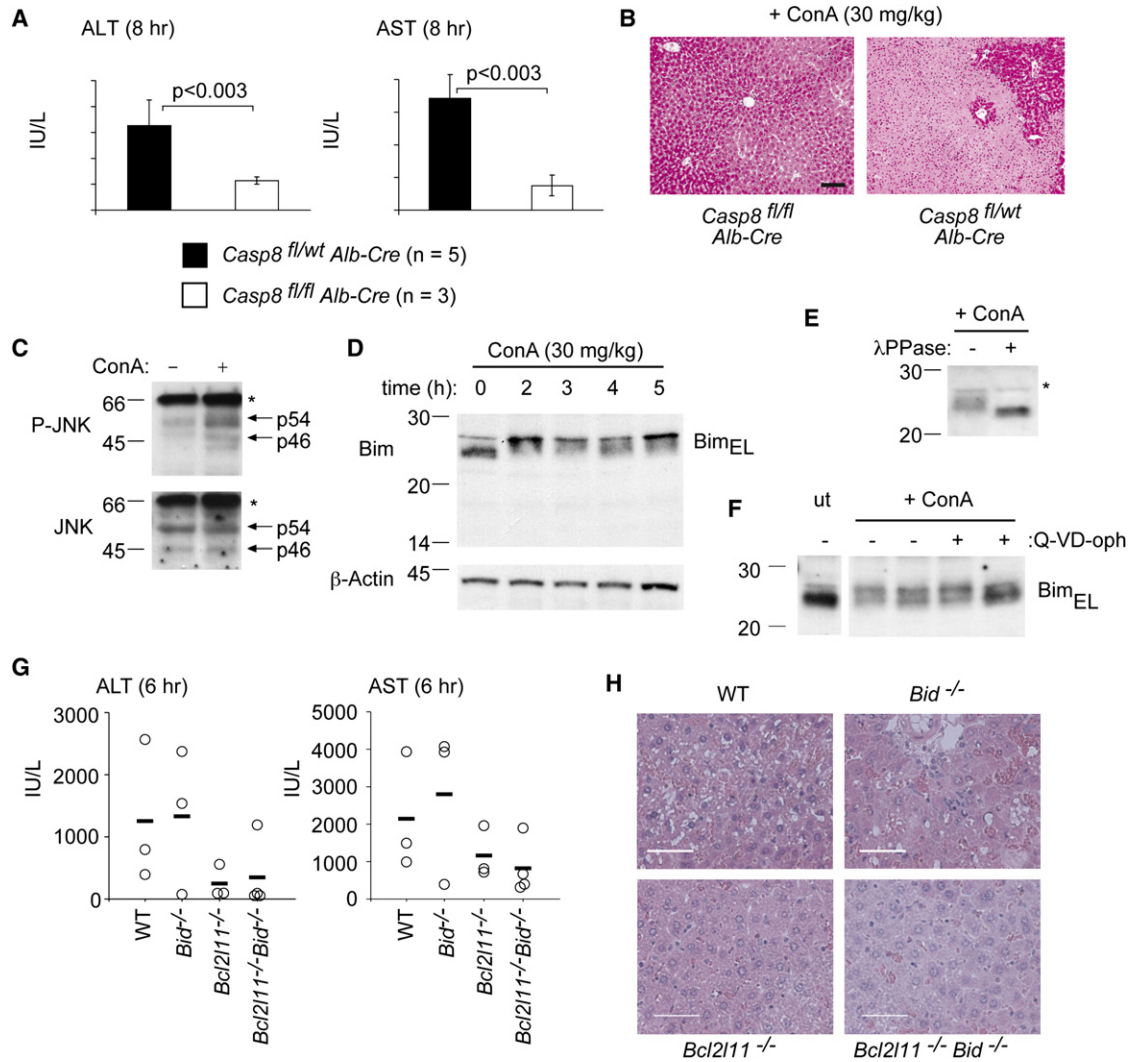


Figure 7. Caspase-8 Is Essential and Bim a Contributor in ConA-Induced Hepatitis

(A) Mice lacking caspase-8 in hepatocytes (*Albumin-Cre* transgenic *Casp8^{fl/fl}*) and littermate controls (*Albumin-Cre* transgenic *Casp8^{fl/wt}* mice) were injected with 30 mg/kg ConA. At the time when the control mice were sick (8 hr), all animals were sacrificed and serum levels of ALT and AST were measured. Data shown represent means \pm SD of three to five mice for each genotype and each treatment.

(B) H&E-stained histological liver sections from mice treated as in (A). Pictures shown are representative of the analysis of at least three mice for each treatment and genotype.

(C) Liver extracts from WT mice treated with 30 mg/kg ConA for 4 hr were probed by immunoblotting with antibodies specific for phosphorylated (activated) JNK or total JNK (loading control).

(D) WT mice were injected with ConA (30 mg/kg) and sacrificed at the time points indicated. Bim levels and possible posttranslational modifications were analyzed by immunoblotting of liver extracts as described in Figure 5A.

(E) WT mice were injected with 30 mg/kg ConA and sacrificed after 3 hr. Total protein extracts were prepared from the liver in the absence of phosphatase inhibitors and left untreated or treated *in vitro* with λ -phosphatase prior to analyzing Bim modifications by immunoblotting.

(F) WT mice, with or without pretreatment with 20 mg/kg of the pan-caspase inhibitor Q-VD-oph, were injected with ConA (30 mg/kg) and sacrificed 5 hr later. Total protein extracts from the livers of these animals were probed by immunoblotting for Bim.

(G) Mice of the genotypes indicated were injected with 30 mg/kg ConA. At the time when the WT mice were sick (6 hr), all animals were sacrificed and serum levels of ALT and AST were measured. Each circle represents measurements from an individual mouse. Horizontal bars indicate the mean.

(H) Histological examination of H&E-stained liver sections of mice of the indicated genotypes subjected for 6 hr to treatment with 30 mg/kg ConA (scale bars represent 50 μ m). Pictures shown are representative of the analysis of at least three mice for each genotype.

Cook University, Townsville, Australia. C57BL/6 *perforin^{-/-}* mice (Kägi et al., 1994) were generated with C57BL/6 ES cells. C57BL/6 *GranzymesABM^{-/-}* mice were established by intercrossing C57BL/6 *GranzymeM^{-/-}* mice with C57BL/6 *GranzymesAB^{-/-}* mice (Pao et al., 2005) (both strains generated

with 129Sv ES cells and backcrossed to C57BL/6 for >12 and 8 generations, respectively). All experiments with mice were performed according to the guidelines of the animal ethics committees of the Melbourne Health Research Directorate or the Ontario Cancer Institute.

In Vivo Models of Hepatitis

For Fas-mediated hepatitis, mice were injected intravenously (i.v.) with 0.25 $\mu\text{g/g}$ body-weight recombinant soluble Fas ligand (FLAG-tagged, Apotech) that had been crosslinked with 2 μg FLAG antibody (M2, SIGMA) per μg of FasL. For the LPS+GalN model, mice were injected intraperitoneally (i.p.) with 10, 100, or 1000 ng LPS (DIFCO) in the presence of 20 mg of the liver-specific transcriptional inhibitor D-(+)-galactosamine (GalN, SIGMA). For T cell-activation-mediated hepatitis, mice were injected i.v. with 30 mg/kg body weight of ConA (SIGMA). For biochemical studies, at the time when WT mice succumbed to these treatments, all mice of an experimental group were sacrificed and bled (for serum analysis of liver enzymes), and the livers were removed for histological analysis. For survival analysis, mice were treated as indicated and monitored until becoming terminally ill. Statistical analyses were performed by applying a two-tailed unpaired t test. The pan-caspase inhibitor Q-VD-oph (MP Biomedicals) was administered i.p. at 20 mg/kg, 30 min prior to LPS+GalN injection. The D-JNK11 inhibitory peptide (H-Gly-D-Arg-D-Lys-D-Lys-D-Arg-D-Arg-D-Gln-D-Arg-D-Arg-D-Arg-D-Pro-D-Pro-D-Arg-D-Pro-D-Lys-D-Arg-D-Pro-D-Thr-D-Thr-D-Leu-D-Asn-D-Leu-D-Phe-D-Pro-D-Gln-D-Val-D-Pro-D-Arg-D-Ser-D-Gln-D-Asp-D-Thr-NH₂) (GL Biochem Shanghai Ltd) was administered i.p. at 30 mg/kg, 30 min prior to LPS/GalN injection.

Immunoblotting and Caspase-Activity Assays

Mouse livers were surgically removed and cell suspensions were prepared by passing them through a stainless-steel sieve. Red blood cells were lysed in a hypotonic buffer and hepatocyte lysates prepared in a buffer containing 20 mM Tris/HCl (pH 7.4), 135 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 500 $\mu\text{g/mL}$ Peabloc (AEBSF), 1 $\mu\text{g/mL}$ Leupeptin, 1 $\mu\text{g/mL}$ Aprotinin, 1 $\mu\text{g/mL}$ Pepstatin, 100 $\mu\text{g/mL}$ soybean trypsin inhibitor, and 2 $\mu\text{g/mL}$ E64. Proteins (40 μg) in cell lysates were size-separated on precast 12% SDS PAGE gradient gels (Invitrogen). Membranes were probed with a rat Bid monoclonal antibody (clone 2D1, Alexis; Kaufmann et al., 2007b), a polyclonal rabbit Bim antibody (Stressgen), a rat anti-mouse caspase-8 monoclonal antibody (clone 1G12, Alexis; O'Reilly et al., 2004), a rabbit polyclonal antibody to active caspase-3 (Cell Signaling), a mouse monoclonal antibody to caspase-7 (gift from Y. Lazebnik), a rat monoclonal antibody to c-FLIP (clone Dave-2, Alexis), a rabbit polyclonal antibody to Mcl-1 (Rockland), a mouse monoclonal antibody to phospho-JNK, or a rabbit polyclonal antibody to JNK (both from Cell Signaling). Probing with a mouse monoclonal antibody to β -actin (SIGMA, AC-40) served as a loading control.

A fluorogenic assay for effector-caspase activity in liver extracts was performed according to the manufacturer's (Bachem, Switzerland) protocols.

Subcellular Fractionation

Subcellular fractionation was performed as described earlier (Puthalakath et al., 1999). In brief, $\sim 5 \times 10^6$ liver cells from mice injected with PBS, GalN, or LPS+GalN were lysed in 500 μl of extraction buffer (0.05 M PIPES-NaOH, 0.05 M HEPES [pH 7.0], 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT plus protease, and phosphatase inhibitors) containing 1% Triton X-100. Cell debris and nuclei were removed by centrifugation at 500 \times g. The supernatant was spun at 125,000 \times g for 60 min at 4°C and the filamentous actin-enriched pellet (P1) was discarded. The remaining supernatant was incubated for 13 min at 37°C with 20 μM taxol and 5 U apyrase (Sigma). This mixture was gently laid on top of 0.5 ml 7.5% sucrose (in extraction buffer) and centrifuged for 30 min at 125,000 \times g at 30°C. The sedimented pellet was saved as the dynein-enriched P2 fraction and the supernatant as the S fraction.

SUPPLEMENTAL DATA

Supplemental Data include nine figures and can be found with this article online at [http://www.immunity.com/supplemental/S1074-7613\(08\)00543-8](http://www.immunity.com/supplemental/S1074-7613(08)00543-8).

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