Genetic Delineation of the Pathways Mediated by Bid and JNK in Tumor Necrosis Factor-α-induced Liver Injury in Adult and Embryonic Mice

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Tumor necrosis factor-α (TNFα)-induced hepatocyte death and liver injury can be mediated by multiple mechanisms, which could be evaluated by different animal models. Previous studies have defined the importance of Bid in mitochondrial apoptosis activation in adult mice treated with lipopolysaccharides in the presence of galactosamine (GalN), which suppresses NF-κB activation, but not in embryonic mice in which NF-κB activation is suppressed by genetic deletion of p65RelA. JNK has also been found important in TNFα-induced mitochondria activation and liver injury in the lipopolysaccharide/GalN and concanavalin A (ConA)/GalN models, but not in a ConA-only model in which NF-κB activation was not suppressed. To determine the mechanistic relationship of pathways mediated by Bid and JNK, we investigated these two molecules in TNFα injury models that had not been previously examined. Most importantly, we created and studied mice deficient in both Bid and JNK. We found that, like JNK, Bid was also required for TNFα-induced injury induced by concanavalin A/GalN but not by ConA alone. Furthermore, our results indicate that these two molecules function in a largely overlapped manner, with Bid being downstream of JNK in the adult livers. However, JNK, but not Bid, was able to contribute to the TNFα-induced liver apoptosis in RelA-deficient embryos. The Bid-independent role of JNK was also observed in the adult mice, mainly in the promotion of the lethal progression of the TNFα injury. This work defined both linear and parallel relationships of Bid and JNK in TNFα-induced hepaticocyte apoptosis and liver injury.

TNFα could induce multiple prodeath mechanisms. The classic mechanism is the activation of the extrinsic apoptosis pathway, in which the engagement of TNF-R1 (tumor necrosis factor receptor 1) recruits the adaptor molecules, TRADD and FADD, which in turn recruit caspase-8. The activation of caspase-8 leads to the cleavage of a prodeath Bcl-2 family protein, Bid, which then triggers the mitochondria apoptosis pathway (1). Previous studies have established that Bid, a BH3-only prodeath Bcl-2 family protein, plays an important role in death receptor-mediated hepatocyte apoptosis and liver injury (2–7). Specifically, deletion of Bid leads to a complete protection against Fas-mediated liver injury. However, Bid-deficient mice are only partially protected from TNFα-mediated liver injury in a model using LPS and GalN as the stimulation (5, 6).

TNFα-induced cell death is suppressed by concurrently activated NF-κB-mediated prosurvival events (8). The protection could be abolished by a general transcription inhibitor, such as actinomycin D or GalN, or by a specific deletion of a key element in the NF-κB pathway, such as p65RelA. RelA is the critical component of the p65-p50 NF-κB dimers that are responsible for the prosurvival mechanisms, including the suppression of caspase and sustained JNK activation (8–10). Deletion of RelA leads to defective NF-κB signaling, unrestrained TNFα toxicity, severe hepatocyte apoptosis, and embryonic lethality (11). The liver injury and lethality could be rescued by a concomitant deletion of TNFα (12), indicating that TNFα is the trigger of the demise. Deletion of Bid, however, was not able to suppress hepatocyte apoptosis and embryonic lethality caused by the deletion of p65RelA (6, 13).

There are several possibilities that may account for the failure of Bid deficiency to protect TNFα-induced liver injury. One may relate to the form of TNFα presented to the receptors. In the model of LPS/GalN, TNFα is mainly produced by macrophages and Kupffer’s cells stimulated by LPS, whereas GalN sensitizes hepatocytes. Notably, TNFα generated in this manner is mainly secreted. It has been found that soluble TNFα primarily activates TNF-R1, whereas membrane TNFα activates both TNF-R1 and TNF-R2 (14). Since the two TNF receptors differ in their ability to activate the death pathway and the NF-κB pathway, it is possible that the membrane and soluble TNFα could induce cell death with different features, as manifested in the different models of TNFα injury (15–18). It is thus also possible that Bid-deficient mice might be more resistant to membrane TNFα in terms of liver injury (e.g. following the treatment with ConA or ConA/GalN).
Differential Roles of Bid and JNK in Liver Injury

Another possibility is that additional death mechanisms other than Bid are activated by TNFα receptors. Our earlier studies indicate that JNK, reactive oxygen species, and mitochondria permeability transition could contribute to the Bid-independent mechanisms in TNFα-induced cell death (6). JNKs have been long considered to be important for TNFα toxicity in both hepatocytes and nonhepatocytes (for reviews, see Refs. 9 and 10). Sustained JNK phosphorylation is correlated with TNFα toxicity and is observed when NF-κB activation is suppressed. Although there is a redundancy of JNK1 and JNK2, deletion of either could significantly block TNFα-induced death in hepatocytes in vitro (19, 20) and in vivo (18, 21–23).

Here we determined the relationship of the Bid-mediated pathway and the JNK-mediated pathway by examining mice deficient in Bid, JNK2, or both in several TNFα injury models where TNFα can be presented in soluble or membranous form.

**EXPERIMENTAL PROCEDURES**

**Animals**—Wild-type and Bid-deficient mice were maintained in C57BL/6 background as previously described (2). JNK1-deficient mice (B6.129S1-Mappk8tm1Flv/J) and JNK2-deficient mice (B6.129S2-Mappk9tm1Flv/J) maintained in C57BL/6 background were obtained from the Jackson Laboratory (Bar Harbor, ME). Bid/JNK2 doubly deficient mice were created by intercrossing the Bid-deficient mice with the JNK2-deficient mice. Mice heterozygous for the p65relA gene were maintained in a mixed background of C57BL/6 and 129SvJ as previously described (6, 11). These mice were further intercrossed to jnk1−/−, jnk2−/−, and/or bid−/− mice to create mice deficient or heterozygous for these genes as described under “Results.” Genotypes of the progenies were determined by PCR as previously described (11, 24, 25). Timed breeding was conducted, with the mornings when vaginal plugs were detected as day 0.5 (±0.5). Animal procedures were conducted according to the guidelines of the National Institutes of Health and protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

**Reagents**—The following antibodies were used: anti-caspase-8, anti-caspase-9, anti-caspase-3 (Cell Signaling, Boston, MA), anti-β-actin (Sigma), anti-cytochrome c, anti-Smac, anti-JNK (clone 0111: B4), ConA, and phalloidin were obtained from Sigma (St. Louis, MO). B6.129S1-Mappk8tm1Flv/J mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Bid/JNK2 doubly deficient mice were created by intercrossing the Bid-deficient mice with the JNK2-deficient mice. Mice heterozygous for the p65relA gene were maintained in a mixed background of C57BL/6 and 129SvJ as previously described (6, 11). These mice were further intercrossed to jnk1−/−, jnk2−/−, and/or bid−/− mice to create mice deficient or heterozygous for these genes as described under “Results.” Genotypes of the progenies were determined by PCR as previously described (11, 24, 25). Timed breeding was conducted, with the mornings when vaginal plugs were detected as day 0.5 (±0.5). Animal procedures were conducted according to the guidelines of the National Institutes of Health and protocols approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

**Treatment of Mice**—Mice were treated as previously described (3, 6). Briefly, male or female mice about 20–30 g in weight were intraperitoneally administered with GalN (20 mg/mouse, for LPS study or 700 mg/kg for ConA study). Thirty minutes later, they were intraperitoneally given LPS at 50 μg/kg or intravenously given ConA at 25 mg/kg. All reagents were prepared in 0.9% (v/v) endotoxin-free sterile saline (Sigma). Animals were sacrificed at designated time points for analysis. Survival analysis was based on the time when mice became moribund.

**Preparation of Liver Subfractions**—Liver homogenates were prepared and fractionated as described previously (3, 6). Briefly, each liver was homogenized in 6 ml of Buffer A (200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM HEPS, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.2) with a Wheaten Dounce glass homogenizer (type B pestle) and centrifuged at 4 °C for 10 min at 700 × g. The supernatants were further centrifuged at 10,000 × g for 15 min. The supernatants were collected as the cytosol fraction. For Western blot and caspase activity measurement, liver lysates from individual mice within the same treatment group were pooled to avoid any bias caused by conditions in individual mouse.

**Biochemical Assays**—The serum level of ALT was measured using the assay kit purchased from Biotron Diagnostics (Hemet, CA) according to the manufacturer’s instructions.

For immunoblot analysis, liver cytosol (80 μg) was separated by a 12% SDS-PAGE followed by transfer to polyvinylidene difluoride membranes and blotted with various antibodies as indicated. Blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce).

Caspase activities were measured as previously described (3, 6). Briefly, 15 μg of cytosol were incubated with 20 μM site-specific tetrapeptide substrates (Ac-DEVD-AFC for caspase-3, Ac-IETD-AFC for caspase-8, and Ac-LEHD-AFC for caspase-9; Calbiochem) in a caspase assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM dithiothreitol, 1 mM EDTA, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, pH 7.2) in a final volume of 200 μl. The fluorescence signals were detected by a fluorometer (Tecan GENios) at an excitation wavelength of 400 nm and emission wavelength of 510 nm.

**Histology**—Livers were dissected, fixed in 10% neutral buffered formalin, and paraffin-embedded. Sections were cut at 5 μm in thickness and stained with hematoxylin and eosin. In addition, frozen sections of livers from selected samples were stained with phalloidin and Hoechst 33342 to visualize the parenchymal structures and apoptotic nuclei. Images were obtained using a light microscope (Nikon Eclipse E200, Melville, CA) equipped with a digital camera (SPOT, Diagnostic Instruments, Sterling Heights, MI).

**Statistical Analysis**—All numerical results were expressed as the mean ± S.D. and represent data from a minimum of three independent experiments. The statistical analysis was performed with SigmaStat 3.0 (SPSS Science, Chicago, IL) with a p value of less than 0.05 being considered as a significant difference. The survival rate was analyzed using the Kaplan-Meier method, and significance was determined by log rank analysis using SigmaStat 3.0 (SPSS Science).

**RESULTS**

*Bid Is Important but Not Absolutely Required for the Mitochondria Apoptotic Response and Liver Injury Caused by ConA/GalN Treatment*—To determine whether Bid-deficient mice would respond to membrane TNFα in a way different from that to soluble TNFα, mice were administered with ConA, a T cell mitogen that leads to the increased expression of TNFα on the surface of T cells, which is the cytokine primarily responsible for the liver injury (26, 27). Unlike LPS, ConA alone could induce a reversible and mild liver injury without the sensitization with GalN (27, 28). However, when mice are sensitized with GalN, a significantly more severe injury can be induced with a rapid development of hepatocyte apoptosis, and the
mortality is high (28). Interestingly, Bid-deficient mice seemed to be equally susceptible to ConA-only-induced liver injury as the wild type mice but were significantly protected from the more severe injury caused by ConA/GalN (Fig. 1A and B).

Previous work had indicated that liver injury induced by ConA alone was not primarily caused by a caspase-mediated apoptotic process (23, 28). Consistently, caspase activation was not detected in either wild type or Bid-deficient livers 8 h after treatment when cellular injury was already apparent (Fig. 1C). However, in the presence of GalN, ConA could induce very robust caspase activation (Figs. 1D and 2A). Similar to the LPS/GalN regime, a ConA/GalN regime can induce a significant activation of the mitochondria apoptotic pathway with the release of cytochrome c and Smac around 4–6 h after the treatment (Fig. 1D). This kinetics was well correlated with the caspase activity arising at the same time frame (Fig. 2A). Notably, both the mitochondrial response and caspase activation were greatly diminished in Bid-deficient livers in terms of the kinetics and the severity (Fig. 1D), indicating that Bid, as in other cases of death receptor activation, is also the major molecule mediating the mitochondria apoptotic pathway in the LPS/GalN regime. Bid-independent activation of mitochondria and caspases occurred later (Fig. 1D), much like that observed in the LPS/GalN regime (6).

The apoptotic response apparently dictated the fast progression of the liver injury in ConA/GalN treatment. Consistent with caspase activation, serum levels of ALT, a marker of liver injury, was increased at 4 and 6 h after treatment in wild-type and Bid-deficient mice, respectively (Fig. 2C). Thus, based on serum ALT and liver histology, deletion of Bid attenuated liver injury induced by ConA/GalN. All wild type mice died within 24 h, with a mean survival time of 7.45 h (Fig. 2D and Table 1). Deletion of Bid resulted in a noticeable but not significant prolonged survival time (8.14 h), and all mice still died within 24 h.

These studies indicated that ConA alone initiated a non-caspase-dependent liver injury, as shown before (23, 28), which was not affected by the deletion of Bid. However, in the ConA/GalN scenario, deletion of Bid provided a significant protection against caspase activation and attenuated the injury and mortality. The failure of Bid-deficient mice to be completely protected from ConA/GalN treatment suggested that the Bid-independent mechanism could be activated regardless the form of TNFα, soluble or membranous.

Co-deletion of Bid and JNK2 Revealed both Overlapped and Nonoverlapped Functions of the Two Molecules—We thus went on to search whether additional prodeath molecules could be activated downstream of TNFα signaling that might compensate for the loss of Bid. In our earlier in vitro studies, we found that JNK1 and reactive oxygen species could contribute to the Bid-independent mechanisms (6). Interestingly, mice deficient in JNK2 were found to be significantly protected from LPS/GalN-induced (21) (Fig. S1) or ConA/GalN-induced (23) hepatocyte apoptosis and liver injury. We also found that JNK1-deficient mice had similar phenotypes, although less significant than JNK2-deficient mice (23) (Fig. S1). To further determine the relationship of the signaling pathways mediated by Bid and JNK, we established mice deficient in both Bid and JNK2 and compared their response with that of the singly deficient mice in the two models of liver injury.
Differential Roles of Bid and JNK in Liver Injury

**FIGURE 2.** Single or combined deletion of Bid and JNK2 confers significant protection against ConA/GalN-induced liver injury and mortality. A, wild-type (+/ +; solid columns), Bid-deficient (Bid+/−; open columns), JNK2-deficient (JNK2−/−; crossed columns), and Bid/JNK2 doubly deficient (Bid/JNK2−/−; gray columns) mice were either untreated or treated with ConA/GalN and sacrificed at 2, 4, 6, and 8 h later. Mice were sacrificed, and the liver cytosol prepared, which was subjected to the measurement of caspase-3 activities (A) and immuno blot analysis with antibodies (B; 6 h after treatment). Blood was also collected, and the serum level of ALT was determined (C). For A and C, results (mean ± S.D.) were from at least three mice for each group at the indicated time points. *, p < 0.05, compared with the wild type group. D, Kaplan-Meier survival analysis of wild-type (+/ +, n = 7), Bid−/− (n = 15), JNK1−/− (n = 10), JNK2−/− (n = 13), and Bid/JNK2−/− (n = 11) mice following ConA/GalN treatment. p values indicated for each group were based on comparisons with the wild type group (log rank analysis). JNK1−/−, JNK2−/−, and Bid/JNK2−/− mice were significantly resistant to ConA/GalN.

**TABLE 1** Survival analysis of mice with different Bid and JNK genotypes in TNFa-induced liver injury

n is the number of mice used in each group. Survival refers to the percentage of mice that survived in the first 24-h period. Mean survival time and S.E. were determined by the Kaplan-Meier method.

| Parameter | Genotype | ConA/GalN treatment | LPS/GalN treatment |
|-----------|----------|---------------------|---------------------|
|           |          | n | Survival | Mean survival time (h) | S.E. | p | n | Survival | Mean survival time (h) | S.E. | p |
|           | +/ +     | bid−/− | jnk1−/− | jnk2−/− | bid/jnk2−/− | +/ + | bid−/− | jnk1−/− | jnk2−/− | bid/jnk2−/− |
| ConA/GalN |           | 7  | 15  | 10  | 13  | 11  | 7  | 15  | 10  | 13  | 11  |
| n         |          | 8.00 | 0.00 | 0.00 | 0.00 | 0.00 | 8.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Survival (%) |          | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 |
| Mean survival time (h) |          | 7.453 | 14.144 | 10.717 | 13.154 | 13.263 | 6.4  | 7.783 | 9.616 | 15.007 |
| S.E.      |          | 0.351 | 0.39  | 1.568 | 1.754 | 2.144 | 0.202 | 0.226 | 1.46  | 1.46  | 2.092 |
| p         |          | 0.05  | 0.029 | 0.0026 | 0.0006 | 0.0002 | 0.579 | 0.0004 | 0.0023 | 0.0001 | 0.0001 |
| vs        |          | 0.0366 | 0.375 | 0.843 |
| vs        |          | 0.0123 | 0.035 |

* p values were determined by log rank analysis of the comparisons between the specific groups and the group indicated with “vs.” The values in bold have reached the significance level.

The results suggested that the Bid-mediated pathway and the JNK2-mediated pathway were largely but not completely overlapped. Bid deletion had a more dramatic impact than JNK2 deletion on caspase activation and mitochondrial release of apoptogenic factors at the early time point (5–6 h) after ConA/GalN (Fig. 2, A and B) or LPS/GalN (Fig. 3, A and B) stimulation. Co-deletion of Bid and JNK2 did not result in a stronger inhibition than deletion of Bid alone, suggesting that most of the JNK2 effects on caspase activation were mediated by Bid. At the later time point (8 h after treatment), the Bid-independent activation of caspases became more evident, as revealed before (6). However, deletion of JNK2 did not seem to consistently or significantly confer any better protections than Bid deletion alone; nor did the double deletion of Bid and JNK2 (Figs. 2A and 3A). Large variations were observed among individual mice. Overall, the data seemed to indicate that JNK may not play a key role in Bid-independent caspase activation in these in vivo models.

The effects of these two molecules on blood liver enzyme levels were similar. Deletion of Bid, but not JNK2, had noticeable inhibitory effects on blood ALT levels, and combined deletion of Bid and JNK2 did not further reduce the level (Figs. 2C and 3C), suggesting that Bid was mainly responsible for the liver damage and that most of the injury was probably caused by Bid-mediated caspase activation, which could overlap with that mediated by JNK.

Paradoxically, deletion of JNK1 or JNK2 conferred a greater protection against mortality than deletion of Bid, with JNK2 deletion being more significant (Figs. 2D and 3D and Table 1). However, co-deletion of JNK2 and Bid resulted in the best protection, particularly in the LPS/GalN models. In the ConA/GalN model, although the mean survival times were similar between the JNK2-deficient and Bid/JNK2-deficient mice (13.15 and 13.26 h, respectively), the survival rate was 15.4 and 27.3% (Table 1). These differences were even larger in the LPS/GalN model with 40% of Bid/JNK2-deficient mice surviving and a mean survival time of 15.01 h, whereas 16.7% of JNK2-deficient mice survived, with a mean survival time of 10.86 h. Thus, it seems that although JNK had less impact on caspase activation and blood ALT levels, it had a significantly greater impact on the mortality, suggesting that mortality is not necessarily all caused by Bid-mediated caspase activation and that JNK might have other major effects on the animal survival. From this aspect, JNK and Bid had apparently nonoverlapped functions.

Deletion of JNK1 or Deletion of JNK2 Alone or in Combination with Bid Could Not Fully Rescue TNFα-dependent p65RelA...
A birth ratio close to the expected 0.25 for RelA-deficient embryos could be observed up to E15.5, which was then greatly reduced by E16.5 (Fig. 4A). We could recover some RelA-deficient embryos by E16.5, although they had apparently died by then (Fig. 4B). There were almost no RelA-deficient embryos recovered beyond E16.5 (data not shown). Deletion of JNK1 or JNK2 resulted in an increased recovery of RelA-deficient embryos at E16.5, so that the percentage of RelA-deficient embryos recovered increased from around 16% to around 23% (Fig. 4A).

Morphological examination (Fig. 4B) of the recovered embryos indicated that some of the E14.5 and all of the E15.5 RelA-deficient embryos looked paler than their wild type and heterozygous counterparts, and their livers looked more hemorrhagic, suggesting the onset of liver injury. The internal organs of these embryos were still intact, and the liver could be recovered for further analysis. By E16.5, all RelA-deficient embryos were apparently dead, with reduced, partially absorbed bodies and degrading internal organs. No livers could be recovered. Although a concomitant deletion of JNK1 or JNK2 did not change the type of pathologies observed in E14.5 and E15.5 RelA-deficient embryos, the progression to death in these embryos seemed to be somewhat delayed, based on the better preserved morphology of the recovered embryos at E16.5 (Fig. 4B). This was particularly noticeable for JNK2/RelA doubly deficient mutants. These data seemed to be consistent with the increased number of such embryos recovered at E16.5 (Fig. 4A) and suggested that JNK could play a role in RelA deficiency-induced early embryonic lethality.

Consistent with the morphological finding, only a portion of E14.5 RelA-deficient embryos underwent apoptosis with increased caspase activities in the liver. The caspase activation as a more objective criterion, we found that about 57.1% (12 of 21) of single RelA-deficient embryos, 62.5% (5 of 8) of doubly RelA/JNK1-deficient embryos, and 50% (6 of 12) of doubly RelA/JNK2-deficient embryos had developed the TNFα-induced liver injury. However, RelA-deficient embryos lacking JNK1 or JNK2 tended to have a lower caspase activity, although a large variation could be observed among individual embryos (Fig. 5A). By E15.5, apoptosis progressed significantly in all RelA-deficient embryos no matter whether they expressed JNK or not (n = 7–11/group). Correspondingly, the caspase activities were further elevated, but the difference between wild type and JNK1- or JNK2-deficient embryos remained observable.
Differential Roles of Bid and JNK in Liver Injury

FIGURE 4. Limited suppression of RelA deficiency-induced lethality by JNK1 or JNK2 deletion in the presence or absence of Bid. A, heterozygous RelA-deficient mice carrying no additional genetic defect, or homozygous jnk1 deletion, or homozygous jnk2 deletion, or homozygous bid and jnk2 deletion were intercrossed. The ratio of relA-deficient embryos at E14.5, E15.5, or E16.5 versus all embryos were calculated on the background of rela−/− only (open column), rela/jnk1−/− (gray column), rela/jnk2−/− (solid column), or rela/ jnk2/bid−/− (lined column). The number of embryos analyzed ranges from 22 to 99 per group. The expected percentage of RelA-deficient embryos is around 25% from such breeding if there is no loss of the embryos. The observed percentage is noticeably lower for RelA-deficient embryos at E16.5, which seemed to be improved with the deletion of JNK1 or JNK2 with or without concomitant deletion of Bid. B, representative embryos harvested at E14.5, E15.5, and E16.5 from the above mentioned breeding were shown. E14.5 and E15.5 RelA-deficient (rela−/−) embryos looked paler than their RelA wild type (rela+/+) siblings, and their livers looked hemorrhagic. All E14.5 and E15.5 RelA-deficient embryos shown in this panel had increased caspase activities in their livers. In addition, all recovered E16.5 RelA-deficient embryos seemed to have died with reduced size and deteriorating morphology. Note the apparently better preserved E16.5 Rela−/− dead embryos on the JNK1−/− or JNK2-deficient background with or without Bid, suggesting a delayed death.

Thus, phosphorylated c-Jun could be detected in E15.5 RelA-deficient embryos but not in RelA-positive embryos. Deletion of either JNK1 or JNK2 alone was not sufficient to eliminate the phosphorylation of c-Jun, suggesting that both JNK isoforms could be involved in the process. This seemed to be different from what was observed in the adult mice, in which JNK1 was the major c-Jun kinase following TNFα stimulation (Fig. S1B) (6, 21). It must be pointed out that c-Jun phosphorylation by JNK does not necessarily indicate that c-Jun is the target of JNK for its death-promoting effect.

These findings suggested that deletion of JNK1 or JNK2 alone had only limited impact on RelA deficiency and other mechanisms had to be involved. We thus explored whether additional deletion of Bid would improve the pathology of the RelA-deficient embryos. Early studies indicated that deletion of Bid alone did not seem to have any impact on RelA deficiency-induced caspase activation and lethality (6, 13). However, that might be due to the compensatory effects of JNK. Thus, mice deficient in Bid and JNK2 but heterozygous for RelA were created. Intercross between these mice led to the generation of embryos deficient in all three genes. We found that like JNK2/RelA doubly deficient embryos, Bid/JNK2/RelA triply deficient embryos died around E16.5, and the correct ratio was maintained at least by E16.5 (Fig. 4A). These embryos had a morphological presentation similar to those of the JNK2 and JNK1 single deletion embryos (Fig. 4B). Thus, they survived better than RelA singly deficient embryos (Fig. 4) and Bid/RelA doubly deficient embryos (6). Consistently, although caspase activation could be detected in all of the triple knock-out mice by E15.5, the activity was greatly reduced to the same level as that in the JNK2 single deleted embryos (Fig. 5A). However, it was no lower than that in the JNK2/RelA doubly deficient livers. Thus, the rescue of RelA-deficient embryos was very much like that of ConA/GalN-treated adult mice, in which JNK2 but not Bid played a more important role in mortality. In addition, in this RelA-deficient model, JNK2 also seemed more important than Bid in controlling caspase activation (Fig. 5) (6).

DISCUSSION

TNFα-induced liver injury could be evaluated in several models, including those of LPS/GalN, ConA, ConA/GalN, and RelA deletion. Deletion of RelA leads to embryonic lethality; thus, it is also a model for studying TNFα-induced injury in embryonic livers. The other three models are applied to adult mice only. Previous studies from this laboratory have defined the important role of Bid in the liver injury caused by LPS/GalN but not by RelA deletion (3, 5, 6, 29). We and others have also defined that JNK, particularly JNK2, is important for liver injury caused by LPS/GalN (21) (Fig. 3 and Fig. S1) and by ConA/GalN (23) but not by ConA alone (23), although others found that JNK participated in ConA-alone-induced injury (18). The present study evaluated the contribution of Bid to TNFα-induced liver injury in the ConA and ConA/GalN models and the contribution of JNK1 and JNK2 to TNFα-induced liver injury in the RelA deletion model. Furthermore, we explored the relationship of Bid and JNK-mediated pathways in mice deficient in both Bid and JNK2. By comparing the effects of Bid and JNK in all of the four models of TNFα injury, we aimed to define
the mechanistic relationship of Bid and JNK using various parameters.

As in the LPS/GalN treatment, Bid-deficient mice demonstrated significant resistance to ConA/GalN-induced mitochondria apoptotic response and liver injury. As defined in earlier studies (16–18), a major difference between the two injury paradigms is the cellular origin of TNFα and hence the form of TNFα that binds to the TNFα receptors on the target cells. ConA-stimulated TNFα is from T cells (12, 16, 18, 30) and is mainly in the membrane form, which could bind to both TNF-R1 and TNF-R2, whereas LPS-stimulated TNFα is from macrophages and Kupffer’s cells and is mainly in the soluble form, which primarily binds to TNF-R1 (16–18). Despite the fact that membrane and soluble TNFα may activate certain intracellular signaling pathways to different extents because of the differential involvement of TNF-R2 (18), we have not observed any significant differences in the impact of Bid on mitochondria activation between the LPS/GalN and the ConA/GalN regimes. Deletion of Bid resulted in the arrest of mitochondria apoptotic activation at the early time points (4–6 h), and in both cases a Bid-independent mitochondria and caspase activation arose around 8 h. There were no mice that could survive beyond 24 h. Thus, it does not seem that the Bid-independent cell death is related to the source or the form of TNFα that binds to the hepatocytes.

As shown in earlier studies, ConA alone mainly induces a non-apoptotic and non-caspase-mediated liver injury, which relies on TNFα and other cytokines, such as INFγ (30–32). Consistent with the known molecular targets, deletion of Bid did not
Differential Roles of Bid and JNK in Liver Injury

aff ect this process. This finding was also consistent with our observations that suppression of neither FADD nor JNK conferred protections against this type of injury (23).

When the effects of Bid and JNK are examined in the apoptotic context in the LPS/GalN or ConA/GalN model, it seems that JNK largely works upstream of Bid. In addition, the proapoptotic effects of JNK may not be related to its c-Jun kinase activity. On one hand, c-Jun phosphorylation had not been found to be associated with hepatocyte apoptosis (19). On the other hand, although JNK1 possesses the majority of the c-Jun kinase activity, it is JNK2 that seems to play a more important role in TNFα-induced liver injury. A recent study had found that another JNK kinase target, Itch, is important for JNK1-mediated apoptosis (22). Itch is an E3 ligase that can target cFLIP for ubiquitin-dependent proteasomal degradation. It can be phosphorylated and activated by both JNK1 and JNK2 (33). Thus, JNK2 could activate Itch in the liver following TNFα stimulation, although other possibilities that JNK2 works through a different target could not be ruled out. cFLIP suppresses caspase-8 activation by competitively binding to FADD, and its degradation would thus allow more caspase-8 activation (34). Indeed, we found that FLIP degradation was inhibited in the absence of JNK1 or JNK2 (23). Consistently, caspase-8 activation and Bid cleavage were all reduced (21, 23). In addition, in both ConA/GalN and LPS/GalN regimes, deletion of Bid seemed to have a more significant impact on mitochondria and caspase activation than JNK deficiency at the early time point (Figs. 2A and 3A), indicating that Bid works downstream of JNK. As the result, simultaneous deletion of both Bid and JNK2 did not lead to further suppression, compared with Bid deletion alone. Taken together, these data suggest that JNK and Bid could work in a linear pathway, and Bid activation can be alone. Taken together, these data suggest that JNK and Bid could work in a linear pathway, and Bid activation can be

![FIGURE 6. A working model for the interactions between Bid and JNK-mediated pathways in TNFα-induced liver injury and mortality. TNFα-induced apoptosis is mediated by caspase-8, which cleaves both downstream effector caspase-3 and Bid. In the Type II cells, such as the hepatocytes, cleavage of Bid is important for effective caspase-3 activation via the mitochondrial pathway. JNK, particularly JNK2, is also important for TNFα-induced apoptosis. Its effect may be primarily mediated by its promotion of FLIP degradation, which in turn allows effective caspase-8, and therefore Bid, activation. Thus, the proapoptosis activity of JNK2 is mainly mediated by Bid. Studies on mice deficient in Bid or in JNK2 or in both support the notion that Bid works downstream of JNK2 but also suggest that JNK could contribute to some Bid-independent mitochondrial activation (6). Other Bid-independent mechanisms of mitochondria activation could include reactive oxygen species (6). In addition, JNK2 and JNK1 apparently have a greater role in regulating the mortality of injured mice, which can be independent of Bid. The relative contribution of Bid versus JNK to mortality may also be affected by how the liver injury is induced in different model systems, with Bid playing minimal roles in the embryonic model of RelA deficiency but some roles in the adult models. Finally, a major determinant of mortality could be the necrotic activity of TNFα.

![Diagram of the interactions between Bid and JNK-mediated pathways in TNFα-induced liver injury and mortality.

Blood infiltration into the liver parenchymal is obvious in these cases (Fig. 1A) (3, 23) and is probably caused by the destruction of the endothelial cells as part of the inflammation and cytokine response (37). These effects could be more dependent on JNK than on Bid and may not be related to caspase activation (Fig. 6). Thus, JNK-deficient mice could be better protected. Another major mortality factor that would become important in the absence of Bid and/or JNK is the necrotic and/or autophagic liver injury that might account for some or all of the Bid/JNK2-independent death. This possibility needs to be further addressed in future studies. Finally, we cannot rule out that ConA/GalN or LPS/GalN treatment could have unknown impact on other organ systems, where Bid and/or JNK is also deleted. The individual contribution of these factors to mortality could be different in the LPS/GalN and ConA/GalN stimulations, due to factors such as the different cytokine profiles under the two conditions (30–32).

The better protection against the mortality by JNK in TNFα-induced injury is also evident in the RelA deficiency model. Co-deletion of JNK1 or JNK2 together with RelA notably delayed the death and/or the absorption of the embryos, although the rescue seems to prolong the survival by less than a day. Although most RelA-deficient embryos would die and become absorbed at E15.5–E16.5, more JNK1/RelA or JNK2/
ReLA-deficient embryos were preserved at E16.5 with a better morphology. On the other hand, co-deletion of Bid did not seem to have any positive impact in improving embryo survival (6) (data not shown). Biochemically, co-deletion of JNK, particularly JNK2, was also more effective than co-deletion of Bid in reducing caspase activation in the ReLA-deficient liver, and combined deletion of Bid and JNK2 did not yield more protection than JNK2 deletion alone. From this point of view, the rescue of embryonic lethality by JNK deletion is correlated with the modulation of caspase activation, and for some yet to be defined reasons, Bid does not seem to be involved in these processes at all.

The deletion of JNK1 or JNK2 did not alter the onset of caspase activation and apoptosis in ReLA-deficient livers but only the severity of the process. Deletion of JNK1 also moderately retarded the death of Ikkβ-deficient embryos to a similar extent (22). The failure of a complete and effective rescue may be due to the overlapping function of JNK1 and JNK2. This assumption could be tested theoretically by a concomitant deletion of both JNK isoforms. However, JNK1/JNK2 doubly deficient embryos prematurely die at about the same gestation time as the ReLA-deficient embryos (38). Thus, although it is possible to create a JNK1/JNK2/ReLA triple deficient strain, the embryos may still die even if the ReLA deficiency could be corrected by JNK1/2 deficiency, due to other defects caused by the latter at the same critical time point. Incidentally, this role of JNK in embryo development and adult mice reflects its dual functions in cell death and cell survival and the complex interactions with other signaling pathways.

Although these studies suggest that TNFα-induced apoptosis in adult livers and in ReLA-deficient embryonic livers could share some common mechanisms, in which JNK, particularly JNK2, plays important roles, the apparently insufficient suppression of apoptosis in the ReLA-deficient embryos by single JNK deletion suggests that there could be important differences between the two systems, which may not be simply attributed to the redundant functions of JNK1 and JNK2. This consideration is supported by the observations that neither deletion of Bid (6, 13) nor overexpression of Bcl-2 (39) could suppress hepatocyte apoptosis in the ReLA-deficient embryos, whereas both maneuvers could lead to a significant protection against TNFα-induced liver injury (6, 40) (this study).

In summary (Fig. 6), using a genetic approach, we studied and compared the role of Bid and JNK in various models of TNFα-induced apoptosis and liver injury. Although these two molecules function in a largely overlapped manner with Bid being downstream of JNK in the adult livers, JNK but not Bid, seems to contribute more to the TNFα-induced apoptosis in ReLA-deficient embryonic livers. The Bid-independent role of JNK could also be observed in adult mice, mainly in the promotion of the irreversible and lethal progression of the injury.

REFERENCES
1. Gross, A., Yin, X. M., Wang, K., Wei, M. C., Jockel, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. (1999) J. Biol. Chem. 274, 1156–1163
2. Yin, X. M., Wang, K., Gross, A., Zhao, Y., Zinkel, S., Klocke, B., Roth, K. A., and Korsmeyer, S. J. (1999) Nature 400, 886–891
3. Zhao, Y., Li, S., Childs, E. E., Kuharsky, D. K., and Yin, X.-M. (2001) J. Biol. Chem. 276, 27432–27440
4. Li, S., Zhao, Y., He, X., Kim, T.-H., Kuharsky, D. K., Rabinovich, H., Chen, J., Du, C., and Yin, X.-M. (2002) J. Biol. Chem. 277, 26912–26920
5. Zhao, Y., Ding, W. X., Qian, T., Watkins, S., Lemasters, J. I., and Yin, X. M. (2003) Gastroenterology 125, 854–867
6. Chen, X., Ding, W. X., Ni, H. M., Gao, W., Shi, Y. H., Gambotto, A. A., Fan, J., Berg, A. A., and Yin, X. M. (2007) Mol. Cell. Biol. 27, 541–553
7. Kaufmann, T., Tai, L., Ekert, P. G., Huang, D. C., Norris, F., Lindemann, R. K., Johnstone, R. W., Dixit, V. M., and Strasser, A. (2007) Cell 129, 423–433
8. Kucharczak, J., Simmons, M. J., Fan, Y., and Gelinas, C. (2003) Oncogene 22, 8961–8982
9. Lin, A. (2003) BioEssays 25, 17–24
10. Schwabe, R. F., and Brenner, D. A. (2006) Am. J. Physiol. 290, G583–G589
11. Berg, A. A., Sha, W. C., Bronson, R. T., Ghosh, S., and Baltimore, D. (1995) Nature 376, 167–170
12. Doi, T. S., Marino, M. W., Takahashi, T., Yoshida, T., Sakakura, T., Old, L. J., and Obata, Y. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2994–2999
13. Kaufmann, T., Gugayagan, R., Gerondakos, S., Dixit, V. M., and Strasser, A. (2007) Cell Death Differ. 14, 637–639
14. Grell, M., Douni, E., Wajant, H., Lohden, M., Claus, M., Maxeiner, B., Georgopoulos, S., Lesslauer, W., Kollias, G., Pfizenmaier, K., and Scheurich, P. (1995) Cell 83, 793–802
15. Gantner, F., Kusters, S., Wendel, A., Hatzelmann, A., Schudt, C., and Tiegs, G. (1997) J. Pharmacol. Exp. Ther. 280, 53–60
16. Kusters, S., Tiegs, G., Alexopoulou, L., Pasparakis, M., Douni, E., Kunstle, G., Bluethmann, H., Wendel, A., Pfizenmaier, K., Kollias, G., and Grell, M. (1997) Eur. J. Immunol. 27, 2870–2875
17. Nowak, M., Gains, G. C., Rosenberg, J., Minter, R., Bahjat, F. R., Rectenwald, J., MacKay, S. L., Edwards, C. K., III, and Moldawer, L. L. (2000) Am. J. Physiol. 278, R1202–R1209
18. Maeda, S., Chang, L., Li, Z. W., Luo, J. L., Leffert, H., and Karin, M. (2003) Immunity 19, 725–737
19. Schwabe, R. F., Uchimami, H., Qian, T., Bennett, B. L., Lemasters, J. J., and Brenner, D. A. (2004) FASEB J. 18, 720–722
20. Liu, H., Lo, C. R., and Czaja, M. I. (2002) Hepatology 35, 772–778
21. Wang, Y., Singh, R., Leftkowitch, I. H., Rigoli, R. M., and Czaja, M. J. (2006) J. Biol. Chem. 281, 15258–15267
22. Chang, L., Kamata, H., Solinas, G., Luo, J. L., Maeda, S., Venuprasad, K., Liu, Y. C., and Karin, M. (2006) Cell 124, 601–613
23. Ni, H. M., Chen, X., Ding, W. X., Schuchmann, M., and Yin, X. M. (2008) Ann. J. Pathol. 173, 962–972
24. Dong, C., Yang, D. D., Wysh, M., Whitmarsh, A. J., Davis, R. J., and Flavell, R. A. (1998) Science 282, 2092
25. Yang, D. D., Conze, D., Whitmarsh, A. J., Barrett, T., Davis, R. J., Rincon, M., and Flavell, R. A. (1998) Immunity 9, 575–585
26. Gantner, F., Leist, M., Ilgj, S., Germann, P. G., Freudenberg, M. A., and Tiegs, G. (1995) Gastroenterology 109, 166–176
27. Trautwein, C., Rakemann, T., Brenner, D. A., Streeck, K., Licato, L., Manns, M. P., and Tiegs, G. (1998) Gastroenterology 114, 1035–1045
28. Kunstle, G., Hentze, H., Germann, P. G., Tiegs, G., Meergans, T., and Wendel, A. (1999) Hepatology 30, 1241–1251
29. Ding, W. X., Ni, H. M., DiFrancesca, D., Stolz, D. B., and Yin, X. M. (2004) Hepatology 40, 403–413
30. Gantner, F., Leist, M., Lobse, A. W., Germann, P. G., and Tiegs, G. (1995) J. Hepatology 21, 190–198
31. Hong, F., Juraga, B., Kim, W. H., Radaeva, S., El-Assal, O. N., Tian, Z., Nguyen, V. A., and Gao, B. (2002) J. Clin. Invest. 110, 1503–1513
32. Streeck, K., Fregen, B., Plume, J., Korber, K., Kubicka, S., Sass, G., Bischoff, S. C., Manns, M. P., Tiegs, G., and Trautwein, C. (2001) J. Immunol. 167, 514–523
33. Gao, M., Labuda, T., Xia, Y., Gallagher, E., Fang, D., Liu, Y. C., and Karin, M. (2004) Science 306, 271–275
34. Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, I. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Nature 388, 190–195
35. Tsuruta, F., Sunayama, J., Mori, Y., Hattori, S., Shimizu, S., Tsujimoto, Y., Yoshioka, K., Masuyama, N., and Gotoh, Y. (2004) *EMBO J.* **23**, 1889–1899
36. Yin, X. M., and Ding, W. X. (2003) *Curr. Mol. Med.* **3**, 491–508
37. Lawson, J. A., Fisher, M. A., Simmons, C. A., Farhood, A., and Jaeschke, H. (1998) *Hepatology* **28**, 761–767
38. Kuan, C.-Y., Yang, D. D., Roy, D. R. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1999) *Neuron* **22**, 667–676
39. Gugasyan, R., Christou, A., O’Reilly, L. A., Strasser, A., and Gerondakis, S. (2006) *Cell Death Differ.* **13**, 1235–1237
40. Van Molle, W., Denecker, G., Rodriguez, I., Brouckaert, P., Vandenabeele, P., and Libert, C. (1999) *J. Immunol.* **163**, 5235–5241