Tumor necrosis factor-α (TNFα)-induced cytotoxicity contributes to the pathogenesis in inflammatory and immune responses. Here, we studied the role of pro-death Bcl-2 family proteins and the mitochondria apoptosis pathway in the development of TNFα-induced hepatic injury during endotoxemia. After treating mice with lipopolysaccharide or TNFα in the presence of β-galactosamine, Bid was cleaved and translocated to mitochondria in hepatocytes. Independently, Bax was also activated by the death receptor engagement and translocated to mitochondria. However, its subsequent insertion into the mitochondrial membrane depends on Bid. Nevertheless, Bid was required, but Bax could be dispensable for the mitochondrial release of cytochrome c from mitochondria, suggesting that Bid could activate additional downstream molecules other than Bax. The lack of this Bid-dependent mitochondria activation and cytochrome c release in the bid-deficient mice was responsible for the significantly delayed effector caspase activation and hepatocyte injury upon endotoxin treatment, culminating in a prolonged survival of the bid-deficient mice. Additional genetic factors (1) could further modify the dependence of TNFα toxicity on the mitochondria pathway as the bid-deficient 129/SvJ mice manifested an even higher resistance than the same type of mice in C57BL/6 background. The functional significance of the mitochondria apoptosis pathway was thus elucidated in the TNFα-mediated pathogenesis in vivo.

TNFα is a pleiotropic cytokine whose pathological function is mainly related to the inflammation process (1, 2). This function can be important for the clearance of certain microorganisms, such as Listeria monocytogenes (3, 4), but can also contribute to a number of disease processes, such as endotoxemia and septic shock (5–7). Although some of its pathological effects are due to a series of cytokine reactions, TNFα can also exert direct cytoxic effects on the cell (8–11). The liver is a major in vivo target of TNFα toxicity (12–18). Although the development of the hepatic injury has been shown to be dependent on a caspase-driven apoptotic process (14, 16–19), the precise mechanisms of caspases activation are not clear.

When binding to its 55-kDa receptor TNF-R1, TNFα may activate several different apoptosis pathways based on in vitro studies on cell lines (10, 20–23). The most well studied pathway involves the adaptor molecules TRADD and FADD, which recruit caspase 8, an initiator caspase (23). The activated caspase 8 is then able to activate the effector caspase 3 (23, 24), which, together with other effector caspases, is responsible for many of the destructive cellular events during apoptosis (25). This cytosolic pathway had been considered to be responsible for the death receptor-mediated apoptosis in general (23). Effector caspases, however, can be activated by another pathway in which mitochondria release cytochrome c in response to death signals such as DNA damage or growth factor deprivation. Cytochrome c binds to Apaf-1 to induce its oligomerization, which in turn activates another initiator caspase, caspase 9, which then activates the downstream effector caspases (26, 27). This mitochondria pathway is tightly regulated by the Bcl-2 family proteins. Although the pro-death members of the family, such as Bid, Bax or Bak can promote mitochondrial release of cytochrome c, the anti-death members of the family, such as Bcl-2 and Bcl-xL, can suppress this process (28–30).

The role of the mitochondria-mediated pathway in TNFα toxicity in vivo has not been elucidated. However, several in vitro studies have demonstrated that TNFα could induce cytochrome c release in a variety of cells (31–37), suggesting that TNFα may also be able to induce apoptosis through the mitochondria death pathway. Since Bid, a “BH3-only” pro-death Bcl-2 family protein can be proteolytically activated by caspase 8 in response to death receptor activation, leading to Bid translocation to mitochondria and cytochrome c release (32, 38, 39), we hypothesized that the Bid-initiated mitochondria apoptosis pathway could contribute to the in vivo TNFα cytotoxicity, such as hepatic injury. Indeed, activation of Fas, another death receptor, led to a Bid-dependent hepatic apoptosis in which the activation of effector caspases was significantly dependent on the mitochondria pathway (33). The current study tested this hypothesis and intended to determine the significance and the activation mechanisms of the mitochondria pathway in TNFα-induced hepatic injury. To do so, we selected an animal model employing the bacterial endotoxin, lipopolysaccharide (LPS), and a sensitizing agent, β-galactosamine (GalN) (12, 13, 40). In this model, the activation of caspases in hepatocytes and their roles in the hepatic injury have been well documented (16, 17, 19, 41).

We now report that treatment of C57BL/6 mice with the...
LPS/GalN regime did activate Bid, which was cleaved by caspase 8 to acquire the ability to move to mitochondria. Interestingly, another pro-death Bcl-2 family protein, Bak, was also activated to translocate to mitochondria independently of Bid. However, Bak did require Bid for its subsequent insertion into the mitochondria and therefore for its activity. Activation of Bid seemed to be responsible for the release of cytochrome c and the activation of mitochondria-initiated caspase cascade. Thus both effector caspase activation and liver damage were significantly retarded, although not eliminated, in the absence of Bid. On the other hand, the deletion of the Bax did not seem to significantly affect the mitochondrial release of cytochrome c and caspase activation, suggesting that Bax was not the only molecule that could mediate the activity of Bid. Indeed, a recent study showed that Bid could activate another pro-death Bcl-2 family protein, Bak, by inducing its oligomerization (42), suggesting that Bak could serve as yet another downstream target of Bid. Thus multiple pro-death Bcl-2 family proteins, led by Bid, could be sequentially activated in vivo to trigger the mitochondria apoptotic pathway. Finally, the dependence on Bid to the response to TNFα was even more significant in 129/SvJ mice than in C57BL/6 mice, suggesting that additional genetic factor(s) might further influence the selective use of the mitochondria apoptosis pathway. We thus concluded that pro-death Bcl-2 family proteins and the mitochondria apoptosis pathway played a critical role in the early development of TNFα-induced hepatocyte apoptosis and pathogenesis in vivo.

MATERIALS AND METHODS

Mice—Mice deficient in bid were established by a gene targeting technique (33). These mice have been bred to either C57BL/6J (99% homogeneity) or 129/SvJ (100% homogeneity) background by first crossing the founder chimeric mice to the inbred C57BL/6J or 129/SvJ mice and then continuous back-crossing of the heterozygous mice to the corresponding background. 129/SvJ strain of the Jackson Laboratory is currently named as 129X1/SvJ (Jackson Laboratory, Bar Harbor, ME). Mice deficient in bax were established previously (43) and were maintained in the C57BL/6J background. All mice used in the experiments were male littersmates—25–30 g in weight. They were maintained in a specific pathogen-free facility on the campus in compliance with National Institutes of Health and University of Pittsburgh guidelines.

Induction of Endotoxic Shock—Endotoxic treatments were given as described previously (3, 16, 17, 40). Briefly, mice were given peritoneal injections of lipopolysaccharide (LPS, Escherichia coli serotype 0127: B8, Sigma L-5129) at 25 or 50 μg/kg or n-galactosamine (GalN, Sigma) at 20 mg per mouse. Alternatively, GalN at 700 mg/kg was intraperitoneally administered followed by an intravenous injection of 2 μg/mouse mouse recombinant TNFα (Sigma) 1 h later. All reagents were prepared in 0.9% (w/v) endotoxin-free sterile saline (Sigma). Animals were sacrificed at designated time points for histology and biochemistry studies. Blood plasma was collected for the determination of liver enzyme activities. Staining of liver sections by hematoxylin-eosin or terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) were performed as described previously (33).

Analysis of Cytochrome c Release and Bid Cleavage—Liver homogenates were prepared and subfractionated as described previously (32). Briefly, livers were homogenized in buffer A (200 mm mannitol, 70 mm sucrose, 1 mM EGTA, 10 mM HEPES) with a Wheaton Dounce glass homogenizer (type B pestle) and centrifuged at 4 °C for 5 min at 600 × g. The supernatants were further centrifuged at 7,000 × g for 10 min. The heavy membrane fraction (mitochondria) was washed once again in buffer A by centrifugation at 600 × g for 5 min to remove any contaminating particles and then recovered by centrifugation at 7,000 × g for 10 min. The isolated mitochondria were resuspended in buffer B (250 mm sucrose, 10 mM HEPES-NaOH, pH 7.5, 2 mM KH₂PO₄, 5 mM sodium succinate, 25 μM EGTA) and kept in ice for use within 2 h. Both mitochondrial and cytosol were analyzed by 15% SDS-PAGE followed by Western blotting with an anti-cytochrome c antibody (PharMingen) or anti-Bid antibody (44). Blots were developed by an ECL method (Pierce).

Analysis of the Activation of Bax—To determine the translocation and membrane insertion of Bax, mice were first treated with the LPS/GalN regime or anti-Fas antibodies (clone Jo-2, PharMingen) as described in the figure legends. The liver homogenates and the mitochondria (the heavy membrane fraction) were prepared as described above. One hundred micrograms of mitochondria were treated with freshly prepared 0.1 mM Na₂CO₃, pH 11.5, for 30 min on ice followed by centrifugation at 7,000 × g for 10 min (45). Fifty percent of the recovered pellets, together with an equal amount of untreated mitochondria, were then analyzed by SDS-PAGE followed by Western blotting using an anti-Bax antibody (lot 651) that recognizes mouse Bax at amino acids 43–61 (45, 46).

Analysis of Caspase Activities—Caspase activities were analyzed as described in the previous study (33). Briefly, hepatocyte cytosol (50 μg) was subjected to 15% SDS-PAGE followed by Western blotting with an anti-caspase 8 (Stressgen), anti-activated caspase 3 p19/p17 subunits (Cell Signaling Technology), or anti-14-3-3ε antibody (Santa Cruz Biotechnology). In addition, caspase activities were analyzed by incubating the cytosol (20–30 μg) with 40 μm site-specific tetrapeptide substrates (IETD-AFC for caspase 8 and DEVD-AFC for caspases 3 and 7, Calbiochem) at 37 °C for 30 or 60 min. Fluorescence signals were detected by a fluorescence spectrometer (PerkinElmer Life Sciences LS50-B) at 400 nm excitation and 505 nm emission wavelengths. The signals representative of caspase activities were corrected for background and standardized relative to controls.

RESULTS

LPS and β-Galactosamine Induce Bid Cleavage and Cytochrome c Release in Murine Hepatocytes—One of the most common approaches to induce TNFα-mediated liver damage in mice is to administer a low dose of LPS in the presence of a liver-specific transcription inhibitor, GalN, that results in massive hepatocyte apoptosis (12–14, 16, 17, 19, 40). To test the hypothesis that Bid might be involved in the pathogenesis of this type of liver damage, we first examined whether Bid could be cleaved during such a treatment in hepatocytes and whether cytochrome c was released in correlation to Bid cleavage. Wild-type C57BL/6J mice were intraperitoneally given 50 μg/kg LPS and 20 mg/mouse GalN. Livers were harvested 2 or 4 h later, homogenated, and subfractionated into cytoplasm and heavy membrane (mitochondria) fractions. Western blot analysis of these fractions indicated that Bid cleavage could be detected 2 h following the treatment, but the 15-kDa truncated Bid (tBid) was found only in the cytosol at this time (Fig. 1A).
More cleavage was detected 4 h after the treatment, with more than 50% of tBid now localizing to the mitochondria. Correspondingly, cytochrome c release was detected only at this time when tBid was translocated to mitochondria.

Bid cleavage and cytochrome c release were only observed in mice treated with both LPS and GalN but not in control mice treated with saline or with either reagent alone (Fig. 1B). These findings were consistent with previous reports that liver damage and mortality occurred only when both LPS and GalN were present (40) and suggested that Bid and the mitochondria apoptosis pathway could play a role in the TNFα-mediated hepatocyte apoptosis.

**Bid Mediates the Mitochondrial Release of Cytochrome c in Hepatocytes in Response to LPS or TNFα Treatment**—In order to determine whether cytochrome c release was dependent on Bid, we compared the responses of wild-type and bid-deficient mice to several different treatments. Release of cytochrome c in hepatocytes was observed only in wild-type but not in bid-deficient mice after treatment with either 50 or 25 μg/kg LPS plus GalN (Fig. 2, A and B). This was also true if the mice were directly injected with TNFα plus GalN (Fig. 2C), consistent with the general dogma that the effect of LPS is mediated by the Bid-mediated pathway to the activation of effector caspase 8 (32, 33, 38, 39). The equivalent elevation of caspase 8 activity in both types of mice early in the response was also consistent with the notion that Bid worked downstream of caspase 8 in the death receptor-mediated apoptosis (32, 33, 38, 39).

The activation of caspase 8 led to the cleavage of Bid in wild-type mice under various regimes (Fig. 3C and data not shown). However, effector caspases were only activated in the wild-type, not in the bid-deficient mice, at the early time point (4 h), based on the DEVDase activity assay (Fig. 3D), the detection of the p19/p17 subunits of activated caspase 3, and the cleavage of caspase 3 substrate (14-3-3ε) (Fig. 3, E and F). This was true whether mice were stimulated with LPS or directly by TNFα (Fig. 3D and data not shown). However, effector caspase activities could be detected in bid-deficient livers at a later time point (6 h) after the LPS/GalN treatment (Fig. 3D), although at a lower level than those detected in the wild-type livers. This later activity was apparently not dependent on the mitochondria pathway, since cytochrome c release was not detected, even at this time point in these knockout mice (Fig. 2A). In 129/SvJ mice, which seemed to be more resistant than C57BL/6J mice to this LPS/GalN treatment (see below), the effector caspase activity was also lower than that in C57BL/6J mice at the same time point (Fig. 3D). The contribution of the Bid-mediated pathway to the activation of effector caspase was more significant in these mice, since most 129/SvJ bid-deficient mice had a lower effector caspase activity even at the later time point, 6 h after the treatment (Fig. 3, D and F). This may suggest that the difference in the genetic background could modify the mitochondria-independent caspase activity.

**Fig. 2. TNFα-mediated cytochrome c release in hepatocytes is dependent on Bid.** Wild-type (bid+/+) or bid-deficient (bid−/−) mice of the C57BL/6J (A–C) or 129/SvJ (D) backgrounds were treated as follows. A, 50 μg/kg LPS plus 20 mg of GalN per mouse for 4 or 6 h; B, and D, 25 μg/kg of LPS plus 20 mg of GalN per mouse for 6 h; C, 2 μg/kg TNFα plus 700 mg/kg GalN for 4 h. Liver homogenates were prepared, and the cytosol fractions were isolated. Western blot was conducted with the anti-cytochrome c or anti-β-actin antibodies as indicated. Each lane represents the sample from one mouse.
FIG. 3. Effector caspase activities are significantly reduced in bid-deficient hepatocytes. A, caspase 8 is activated in both wild-type and bid-deficient mice. Wild-type (solid line) and bid-deficient (dashed line) C57BL/6J mice were treated with 50 μg/kg LPS plus 20 mg of GalN per mouse for 4 and 6 h. Liver homogenates were prepared and sub-fractionated. Caspase activities were measured in the cytosol using the peptide substrate IETD-AFC and were normalized to the untreated control. Data represent three experiments. B and C, activation of caspase 8 is independent of Bid. Western blotting using the anti-caspase 8 (B), anti-Bid (C), or anti-β-actin antibody (B and C) was performed on the cytosol from wild-type (bid+/+) and bid-deficient (bid−/−) mice untreated or treated with 50 μg/kg LPS plus GalN for 4 h. Shown were the activated p10 subunit of caspase 8 (B) and the full-length and truncated Bid (tBid) (C). Equivalent amounts of proteins were loaded in each sample as indicated by the anti-β-actin staining. D, caspase 3 activities are much higher in treated wild-type mice than in treated bid-deficient mice. Wild-type (solid bar) or bid-deficient (open bar) C57BL/6J or 129/SvJ mice were treated with 2 μg/kg TNFα or LPS (50 or 25 μg/kg) in the presence of 20 mg of GalN per mouse for 4 or 6 h as indicated. Liver homogenates were prepared, and the cytosol fractions were separated. Caspase activities were measured using the peptide substrate DEVD-AFC and were standardized relative to those of untreated control samples. Data represent 2–4 individual experiments. E and F, early activation of caspase 3 is dependent on Bid. Western blotting was conducted with antibodies against the cleaved p19 and p17 subunits of the activated caspase 3 or the caspase 3 substrate 14-3-3ε on hepatic cytosol prepared from wild-type (bid+/+) or bid-deficient (bid−/−) C57BL/6J mice treated with 50 μg/kg LPS plus 20 mg of GalN for 4 h (E), or on the cytosol of 129/SvJ mice treated with 25 μg/kg of LPS plus 20 mg of GalN for 6 h (F). The cleaved p19 and p17 caspase 3 subunits can be easily observed in treated wild-type mice (E and F), and the p19 subunit was more predominant in the treated C57BL/6 mice (E).
These data indicate that the activity of caspase 3 or 7 was significantly different between the wild-type and \textit{bid}-deficient treated mice. The kinetics of caspase 3 activation was correlated well to those of Bid cleavage and cytochrome \textit{c} release in wild-type mice. Together, it suggests that the early activation of effector caspases in response to TNF\textit{a} was considerably dependent on the mitochondria pathway mediated by Bid.

\textbf{Bax Can Be Independently Activated in Response to LPS/ GalN or Anti-Fas Treatment, but Its Activity Requires Bid—} Recent studies have suggested that Bax, another pro-death Bcl-2 family protein, could be activated by Bid in several cell lines (36, 47–49). To determine whether Bax could be activated \textit{in vivo} to mediate the effect of Bid in response to the LPS/GalN regime, mice were given 50 \textmu g/kg LPS plus GalN for 6 h or 250 \textmu g/kg anti-Fas antibody for 2 h in some cases as described previously (33). Liver homogenates were prepared and sub-fractionated for the analysis.

In the normal hepatocytes, Bax was located almost exclusively in the cytosol. Upon treatment, a significantly larger amount of Bax was found in the hepatocyte mitochondria, indicating that Bax translocation could be stimulated by LPS/GalN or anti-Fas antibodies (Fig. 4, A and B). Surprisingly, this first step of Bax activation was not dependent on Bid, as the translocation also occurred in \textit{bid}-deficient hepatocytes, suggesting that Bax, like Bid, could be independently activated by the death receptor engagement.

However, the second step of Bax activation, \textit{i.e.} the insertion of the molecule into the mitochondrial membranes, was dependent on Bid. The transition of a membrane-attached Bax molecule to a membrane-integrated one was indicated by the acquirement of alkali resistance (Fig. 4B). Peripheral attached molecules can be easily removed by the treatment of the mitochondria with alkali, whereas the integrated molecules are resistant to such a treatment (45). The process of Bax insertion into the membrane was accompanied by a conformational change as suggested by the appearance of a truncated form of Bax (~19 kDa). The truncated Bax most likely resulted from a proteolytic removal of the first 20–30 amino acids at the NH\textsubscript{2} terminus subsequent to its exposure induced by a conformational change (45). Notably, this 19-kDa Bax was not alkali-resistant in contrast to the full-length 21-kDa molecules, suggesting that the proteolysis-sensitive, conformationally changed Bax was an intermediate between the post-translocation membrane attachment form and the membrane integration form. In addition, the amount of this truncated form of Bax varied among experiments, and it was not always detectable (Fig. 4, A and B), suggesting its transient and unstable nature. Furthermore, whereas full-length Bax could be found in the mitochondria isolated from treated \textit{bid}-deficient hepatocytes, it was not alkali-resistant. There was also no detectable truncated form of the molecule in the mitochondria isolated form \textit{bid}-deficient hepatocytes (Fig. 4B). These data indicated that Bid was required for the conformational change of Bax and its subsequent insertion into the membrane.

Since the insertion of Bax into the mitochondrial membrane was required for its activity (45–48), it seemed that if Bax contributed to the development of hepatocyte apoptosis in the LPS/GalN or anti-Fas regime, its activity would be dependent on Bid. On the other hand, the data could also suggest that Bax might serve as the downstream target of Bid and mediate its activity. To test this hypothesis, we examined the response of \textit{bax}-deficient mice to the treatment of 50 \textmu g/kg LPS plus GalN. Cytochrome \textit{c} was found in the cytosol of wild-type as well as \textit{bax}-deficient hepatocytes but not in the cytosol of \textit{bid}-deficient hepatocytes even at 6 h after treatment (Fig. 4C). Correspondingly, caspase 3 activities in the \textit{bax}-deficient mice were also

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4}
\caption{Bax is activated during LPS/GalN treatment but is dispensable for cytochrome \textit{c} release and caspase activation. A, Bax is translocated to mitochondria after LPS/GalN treatment. Wild-type mice were each given either saline or 50 \textmu g/kg LPS plus 20 mg of GalN. Six hours later, the hepatocyte lysates were isolated and sub-fractionated. The presence of Bax in cytosol (Cyto) and mitochondria (Mito) was analyzed by SDS-PAGE followed by Western blotting with an anti-Bax antibody (lot 651). B, Bax insertion into the mitochondrial membrane but not its translocation is dependent on Bid. Wild-type (\textit{bid} +/+ ) or \textit{bid}-deficient (\textit{bid} −/−) mice were each given either 50 \textmu g/kg LPS plus 20 mg of GalN for 6 h or given 250 \textmu g/kg anti-Fas antibody (clone Jo 2) for 2 h. The hepatocyte homogenates were prepared and sub-fractionated. One portion of the mitochondria fraction (100 \mug) was incubated with freshly prepared 0.1x Na\textsubscript{2}CO\textsubscript{3}, pH 11.5, for 30 min on ice, and 50% of the recovered pellet was used for Western blot analysis with an anti-Bax antibody (lot 651) or anti-cytochrome \textit{c} oxidase (Cox) subunit IV (Molecular Probes), a mitochondria marker. An equal amount of control mitochondria not subjected to alkali treatment was analyzed in parallel. C, cytochrome \textit{c} is still released in \textit{bax}-deficient mice in response to TNF/GalN or anti-Fas treatment. Wild-type, \textit{bid}-deficient (\textit{bid} −/−), or \textit{bax}-deficient (\textit{bax} −/−) mice were each given saline (Sal), 50 \textmu g/kg LPS plus 20 mg of GalN for 6 h (L/G), or 250 \textmu g/kg anti-Fas antibody (Fas) for 3 h. The hepatocyte homogenates were prepared, and the cytosol fraction was separated for the detection of cytochrome \textit{c} by Western blot. D, caspase 3 activities are not significantly changed in \textit{bax}-deficient mice in response to LPS/GalN treatment. Wild-type (WT), \textit{bid}-deficient (\textit{bid} −/−), or \textit{bax}-deficient (\textit{bax} −/−) mice were treated with saline (Control) or 50 \textmu g/kg LPS plus 20 mg of GalN per mouse for 6 h. Caspase 3 activities in the liver cytosol were determined using the peptide substrate DEVD-AFC and standardized relative to those of control wild-type mice after a 30- (open bar) or 60-min (solid bar) reaction. Data are representative of three experiments.}
\end{figure}
equivalent to those in the wild-type mice (Fig. 4D). These data indicated that Bid-mediated cytochrome c release and caspase activation could still occur in the absence of Bax, suggesting that Bax was not the only target activated by Bid. These studies were also consistent with previous findings that Bax was dispensable in cytochrome c release and hepatic injury induced by anti-Fas antibody treatment (50).

**Bid-deficient Mice Are More Resistant to Endotoxin-induced TNFα-mediated Liver Damage** — The TNFα-mediated hepatotoxicity induced by LPS/GalN treatment was due to severe hepatocyte apoptosis caused by elevated effector caspase activities (16–19). To evaluate the pathological significance of the Bid-initiated mitochondria pathway in the hepatotoxicity of TNFα, we compared the liver damage and survival rates of the wild-type and bid-deficient mice after treatment (Fig. 5). In the liver sections of wild-type mice given the treatment for 4 h, there were spotted erythrocyte aggregations, which were largely confined in the sinusoids, suggesting that the endothelium was still intact. At this time, there was also no apparent parenchymal damage, although some hepatocytes did show pyknotic nuclei. The liver sections of bid-deficient mice generally exhibited a more healthy appearance, although some red cell aggregation could be detected in the sinusoids as well, possibly reflecting the effect of endotoxins in general.

Six hours after treatment, the wild-type livers demonstrated a significant amount of hepatocytes with apoptotic features, such as pyknotic nuclei (Fig. 5A), which were TUNEL-positive (Fig. 5B). The loss of normal morphology of hepatocytes was accompanied by the destruction of liver parenchymal structure and massive erythrocyte infiltration into the parenchyma, indicating severe damage to the endothelium. In contrast, the liver histology of most bid-deficient mice seemed to be largely normal, with the parenchymal structures maintained and no massive red cell infiltration. Some hepatocytes, which were confined in small, scattered regions, manifested condensed nuclei. But TUNEL staining was generally negative at this stage (Fig. 5B). It has to be pointed out that the liver histology of those bid-deficient mice that eventually died at later time points (around 7–8 h, Table I) became comparable to that of the wild-type mice (data not shown). It seemed that there was an interval about 2 h from peak caspase activation to significant histological changes (Figs. 3 and 5). The delayed pathological change in bid-deficient mice was consistent with the delayed elevation of effector caspase activities (Fig. 3). However, it seemed that this Bid-independent later activity was still effective to induce liver damage under this regime of treatment.

As expected, the serum level of liver enzymes was a more sensitive index for liver injury than the histology. Increased serum lactate dehydrogenase (Fig. 5C) and alanine aminotransferase (data not shown) were detected around 4 h, the time when caspase activities were elevated. The levels were higher in the wild-type mice treated with either LPS (Fig. 5C) or TNFα (data not shown). However, the enzyme levels at 6 h were not significantly different between wild-type and bid-deficient mice, suggesting that this test was less discriminating than the histology examination in the later period.

The mortality was high for mice with the C57BL/6J background (Table I). All wild-type mice entered into the terminal stage in 8 h, with a mean survival time around 7.1 or 6.4 h for the 25 or 50 μg/kg LPS dose group, respectively. In general, C57BL/6J bid-deficient mice survived for a longer time at either dosage (7.8 and 7.3 h, respectively), although the survival rate was equally low. Interestingly, mice with the 129/SvJ background manifested a slower progress of liver damage, as shown by a lower caspase activity (Fig. 3D), lesser tissue damage (data not shown), and prolonged survival (Table II). How-
TABLE I

Comparison of the survival of wild-type and bid-deficient C57BL/6J mice

| LPS | bid +/+ | bid −/− | p* |
|-----|---------|---------|----|
| μg/kg |       |         |    |
| 25   | 7.1 ± 0.7 (n = 12) | 7.8 ± 0.8 (n = 6) | <0.05 |
| 50   | 6.4 ± 0.8 (n = 10) | 7.3 ± 0.5 (n = 4) | <0.05 |

* The difference between bid +/+ and bid −/− was subjected to Student’s t test.

TABLE II

Comparison of the survival of wild-type and bid-deficient 129/SvJ mice

| Genotypes | 0–12 h | 12–24 h | 24–48 h | >48 h |
|-----------|--------|---------|---------|-------|
| bid +/+   | 3/9    | 1/9     | 0/9     | 0/9   |
| bid −/−   | 6/8    | 5/8     | 4/8     | 3/8   |

* The mean survival time of the deceased mice during the first 12 h was 8.53 ± 0.3 (n = 6) for wild-type mice and 9.75 ± 0.25 (n = 2) for bid-deficient mice (p < 0.01 by Student’s t test).

Bid-mediated Pathway in TNFα-induced Hepatocyte Apoptosis

The current studies were designed to investigate how TNFα activated the caspase cascade in an in vivo setting and to examine the mechanisms by which pro-death Bcl-2 family proteins regulated the TNFα response in hepatocytes. We found that the stimulation of TNF-R1 activated the mitochondria apoptotic pathway in a Bid-dependent fashion. Treatment of mice with LPS or TNFα induced Bid cleavage in the hepatocytes. The truncated Bid was then translocated to the mitochondria and induced cytochrome c release. There was no cytochrome c release in treated bid-deficient mice even at later stages, no matter whether these mice eventually developed the liver damage or not. The early release of cytochrome c in wild-type mice seemed critical to the prompt activation of effector caspases in hepatocytes. The kinetics of caspase 3 activation was correlated well with that of Bid cleavage and cytochrome c release in wild-type mice, suggesting that the activation was significantly dependent on the mitochondria pathway at this stage. Furthermore, the effector caspase activities were also correlated well with the elevation of liver enzymes and histology changes, suggesting that the mitochondria pathway mediated by Bid was a major contributing pathway to the liver pathogenesis caused by TNFα. In the absence of Bid, the activation of effector caspases was delayed so that the development of the hepatic injury was impeded, and the survival was significantly improved.

It has to be pointed out that in addition to the Bid-dependent activation of caspases, the Bid-independent death pathway(s) were still active, although less efficient, so that bid-deficient mice, particularly those of C57BL/6 background, could eventually develop the irreversible hepatic injury at a later time point. Modification of the dosage of administered LPS did not seem to alter significantly the overall response in terms of survival or caspase activities, suggesting that the Bid-independent pathway(s) could be activated with minimal signal strength. This is at variance with the anti-Fas-induced hepatocyte apoptosis, in which bid-deficient mice were almost completely resistant to the damage in the proper dose range (33). Thus the Bid-independent phenomenon may not be simply attributed to the potential activation of effector caspases directly by caspase 8, which would be more or less similar to Fas and TNF-R1 activation. Alternatively, other potential death-promoting elements activated by the TNF-R1 could play a role in the hepatocyte apoptosis as well, such as RAIDD/caspase 2 (20, 21), ASK-1 (22), or reactive oxygen species (51). However, all these pathways may not be activated as promptly as the caspase 8-Bid mitochondria pathway, thus making the latter much more significant in the early stage of the development of TNFα toxicity.

Interestingly, the genetic background could further affect the relative weight of the Bid-dependent pathway versus the Bid-independent pathways in the TNFα toxicity. Thus, bid-deficient 129/SvJ mice presented a much reduced caspase activity, mild hepatic damage, and significantly increased survival rate. Although what this genetic influence might be in molecular terms is still ambiguous, it could mean that the Bid-independent caspase activity and/or toxicity are somehow minimized in these mice. Furthermore, it raises the possibility that similar genetic factor(s) may also affect apoptosis induced by other death stimuli in terms of progress and severity.

How does Bid exert its effect to induce cytochrome c release? Bid does not seem to activate the permeability transition pore (42, 50, 52, 53), and it may possess the ability to induce cytochrome c release by itself as it can form channels in vitro (54). Alternatively, it may activate other pro-death Bcl-2 family proteins to mediate its activity. A recent study indicated that Bak could be activated to undergo oligomerization upon the treatment of Bid in vitro or anti-Fas antibodies in vivo in a Bid-dependent manner (42). This process seemed to be required for Bid to induce cytochrome c release at least in vitro. It is conceivable that in the LPS/GalN regime, the activation of Bid could lead to the activation of Bak as in the anti-Fas treatment. Thus, Bak could serve as a downstream target for Bid to mediate its activity.

Bid, however, could activate other pro-death Bcl-2 family
Bid-mediated Pathway in TNFα-induced Hepatocyte Apoptosis

protein as well. In both LPS/GalN and anti-Fas regimes, we found that Bax was activated in the hepatocyte. Although Bax translocation occurred independently of Bid, its subsequent insertion into the membrane was dependent on Bid. The latter was accompanied by a conformational change of Bax as suggested by the appearance of a truncated form of Bax on mitochondrial isolated from the treated wild-type hepatocytes but not on mitochondria isolated from bid-deficient hepatocytes treated equivalently. An earlier study found that mitochondria isolated from apoptotic cells contained a portion of Bax that was susceptible to in vitro trypsin digestion, generating a truncated form of about 19 kDa (45). The digestion likely removed about 20–30 amino acids at the NH2 terminus as the truncated Bax was alkali-resistant in the wild-type mitochondria, and none of the full-length Bax was alkali-resistant in the bid-deficient mitochondria. The truncated Bax observed in the current setting might be generated when some of the Bax undergoing the conformational change on the mitochondrial surface were cleaved by certain intracellular proteases before they were able to insert into the membrane.

Previous studies using in vitro reconstitution systems or cultured cells have observed a mainly consistent phenomenon that Bid can induce Bax conformation change and integration into mitochondrial membranes. However, the role of Bid in Bax translocation from cytosol to mitochondria was not clear. This was due to the use of cell lines that, in most studies, contained a high level of mitochondria-associated Bax, causing a difficulty in recognizing the change in Bax amount in this organelle (36, 37, 45, 47, 49). On the other hand, studies using freshly isolated primary cells or some other cell lines clearly demonstrated the Bax translocation as the first necessary step for its activity (55, 56, 58) but had not assessed any mechanistic differences between translocation and membrane insertion as well as the role of Bid in either step. Thus our studies comparing the response of wild-type and bid-deficient hepatocytes to TNF-R1 or Fas activation provided a unique advantage in assessing the role of Bid in Bax translocation and membrane insertion in an integrated in vivo system. The results for the first time demonstrate that the activation of Bax occurs in at least two steps that can be subjected to different activation signals. In the hepatocytes stimulated through the death receptors, the conformational change and the subsequent membrane insertion depends on Bid, whereas the translocation does not.

Our studies also indicated that death receptor engagement could independently initiate the activation of yet another pro-death Bcl-2 family protein, Bax, upstream of mitochondria. Whereas Bid can be activated through the proteolytic cleavage by caspase 8, the signals downstream of the death receptors to induce Bax translocation are not clear. In thymocytes or neurons subjected to apoptotic stimuli, Bax translocation was associated with an increased intracellular pH (56) or p53 mitogen-activated protein kinase activity (59). Whether the same factors could be responsible for the Bax translocation in the hepatocyte has yet to be determined.

Since Bid participates in the activation of both Bak and Bax, either of them may be sufficient to mediate the effect of Bid. Both Bak and Bax have been shown to possess the ability to oligomerize and to induce cytochrome c (42, 48, 52, 60, 61). Thus it was not surprising that Bax could be dispensed during the Bid-mediated mitochondria release of cytochrome c and hepatic injury in either LPS/GalN (this study) or anti-Fas regime (50). It is likely that Bak could be dispensed as well in the in vivo situation as Bax may then mediate the effect of Bid. This hypothesis is consistent with the recent finding that the combined functions of Bax and Bak are required for the development-regulated apoptosis in mouse, indicating that the two molecules work in parallel pathways and can compensate the loss of one another (62). Thus deletion of both Bak and Bax may be required to achieve the protective effects similar to the Bid deletion in the LPS/GalN or anti-Fas treatment in vivo.
whereas in the in vitro situation deletion of Bak alone could be sufficient to block the activity of Bid (42), as there is normally little Bax on this organelle (Fig. 4A).

In conclusion, our data and those from the literature support a model in which a Bid-led sequential activation of pro-death Bcl-2 family proteins is responsible for the initiation of the mitochondrial apoptotic events in the death receptor-mediated hepatocyte apoptosis (Fig. 6). This pathway is critical in the early activation of caspases in the TNFα or anti-Fas-induced hepatic toxicity so that in the absence of Bid, the apoptotic response is significantly reduced, leading to minor liver damage and improved survival.

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