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TNF Pretreatment Interferes with Mitochondrial Apoptosis in the Mouse Liver by A20-Mediated Down-Regulation of Bax

Gabriele Sass,2* Noula Dattu Shembade,2† Florian Haimerl,*, Nicolas Lamoureux,‡ Said Hashemolhosseini,‡ Andrea Tannapfel,§ and Gisa Tiegs3*

Pretreatment with low doses of the proinflammatory cytokine TNF has been shown to prevent hepatocellular apoptosis and liver damage in inflammatory as well as in ischemia/reperfusion-induced liver injury. The underlying mechanisms of protection have not been elucidated so far. In this study, these mechanisms were investigated in murine hepatocyte cultures as well as in a mouse model of TNF-dependent apoptotic liver damage (galactosamine/TNF model). Our results show that pretreatment with TNF, or application of small-interfering RNA directed against the proapoptotic Bcl2 family member Bax, interfered with the onset of mitochondrial apoptosis in vivo. Knockdown of TNF-α-induced-protein 3 (A20) restored mitochondrial apoptosis, Bax expression, and liver damage. The underlying mechanism of protection seems to involve a cascade of events, where TNF induces the expression of A20 in hepatocytes, A20 down-modulates Bax expression by interference with transcriptional activation, and the reduced availability of Bax interferes with the onset of mitochondrial apoptosis and the ensuing apoptotic liver damage. In conclusion, we identified Bax and A20 as key players in TNF-induced protection from apoptotic liver damage. Because treatment with TNF itself might be a risk factor for patients, we propose that overexpression of A20 might represent an alternative approach for protection from inflammation related apoptotic liver damage, as well as for TNF preconditioning during transplantation. The Journal of Immunology, 2007, 179: 7042–7049.

In liver diseases, such as viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, ischemia/reperfusion injury, or autoimmune hepatitis, activation of the inflammatory response is crucial for damage induction. In most of these cases, acute injury seems to be driven by a proinflammatory Th1 cytokine response. Among these cytokines, TNF, in combination with additional pathophysiologic factors, plays a central role in inducing hepatocyte apoptosis (1, 2). However, TNF is also critical with additional pathophysiologic factors, plays a central role in cases, acute injury seems to be driven by a proinflammatory Th1 tory response is crucial for damage induction. In most of these conditions and dependent on the presence of TNFR1 (10),

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2 G.S. and N.D.S. contributed equally to this work.
3 Address correspondence and reprint requests to Dr. Gisa Tiegs, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Erlangen-Nuremberg, Fahrstrasse 17, D-91054 Erlangen, Germany. E-mail address: gisa.tiegs@pharmakologie.uni-erlangen.de
4 Abbreviations used in this paper: GalN, D-galactosamine; siRNA, small-interfering RNA; HSA, human serum albumin; RU, relative unit; ISEL, in situ end labeling; Act.D, actinomycin D; LDH, lactate dehydrogenase.

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Materials and Methods

Animals

C57BL/6 mice (age: 8–10 wk; weight range: 20–25 g) were obtained from the animal facilities of the Institute of Experimental and Clinical Pathology and Toxicology (University of Erlangen-Nuremberg, Erlangen, Germany). All mice received human care according to the guidelines of the National Institute of Health as well as to the legal requirements in Germany. They were maintained under controlled conditions (22°C, 55% humidity and 12-h day/night rhythm) and fed a standard laboratory chow.

Dosage and application routes

Recombinant murine TNF (Innogenetics), dissolved in saline/0.1% human serum albumin (HSA), was administered to mice i.v. at 10 μg/kg alone or at 5 μg/kg 30 min before n-galactosamine (GalN; Roth) application. GalN was administered i.p. at 700 mg/kg in pyrogen-free saline. For induction of cellular damage, actinomycin D (ActD; 80 ng/ml; Sigma-Aldrich) was added to primary hepatocyte cultures 30 min before TNF (40 ng/ml). The NF-xB inhibitor glutarone (Ref. 21; Alexis Biochemicals) was dissolved in DMSO and diluted to final concentrations in cell culture medium. siRNA was purchased from Eurogentec, Germany. Targets for siRNA design were: AGC GAG TGT CTC CGG CGA ATT for Bax; AAC in inhibitor gliotoxin (Ref. 21; Alexis Biochemicals) was dissolved in DMSO.

Supernatants were centrifuged at 16,000 rpm for 20 min at 4°C to remove nuclei and cell debris. Supernatants were used to perform anti–cytochrome c detection assay. Western blot analysis and control siRNA was applied according to the protocol used for siBax or siA20.

Analysis of liver enzymes and cellular damage

Hepatocyte damage was assessed 6 h after GalN/TNF administration by measuring plasma enzyme activity of alanine aminotransferase (ALT) (23), using an automated procedure. Cellular damage in primary hepatocytes was measured by lactate dehydrogenase (LDH) activity in culture supernatants (5) and the remaining cell monolayer (C) after lysis with 0.1% Triton X-100. The percentage of LDH release (toxicity) was calculated from the ratio of S/(S + C).

Determination of caspase 3 activity

To determine the activation of caspase 3 in liver tissue of mice, liver homogenates (50% w/w) were prepared in lysis buffer containing 10 mM HEPES (pH 7.4), 1 mM CHAPS, and 1 mM DT T and analyzed using the colorimetric caspase 3 Assay kit (Sigma-Aldrich) according to the manufacturer’s instructions.

Western blot analysis

Livers were homogenized in lysis buffer containing 0.5% Nonidet P-40, 137 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl (pH 8.0), 10% glycerol. Following centrifugation, supernatants were stored at −80°C. For Western blot analysis, 20 μg of protein were fractionated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane. Western blots were developed using an ECL system (Amersham) according to the manufacturer’s instructions. Semiquantitative evaluation was done using the Gel Doc 2000 System (Bio-Rad).

Cytochrome c detection assay

To detect cytochrome c release, mitochondria-free cytosolic fractions were prepared as described previously (24). Briefly, tissues were homogenized in ice-cold Mito buffer (20 mM HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 250 mM sucrose, 0.1 mM PMSF, 2 mg/ml pepstatin, leupeptin, and aprotinin) in a small glass homogenizer using a Teflon pestle (50 strokes on ice). The homogenates were centrifuged at 800 rpm for 20 min at 4°C twice to remove mitochondria. Supernatants were used to perform anti-cytochrome c Western blot analysis. A total of 50 μg of protein from the cytosolic fraction were loaded onto a 15% SDS-polyacrylamide gel. Following electrophoresis, gels were blotted onto a 0.2-μm nitrocellulose membrane (Portran; Schleicher & Schuell Bioscience). Western blots were developed as described above.

Antibodies

The following primary Abs were used for immunohistochemical staining and Western blot analysis: anti-A20 (R20), anti-Bax (D21), anti-Bad (G23), anti-Bad (K17), anti-β-actin (C11), and anti-GAPDH (V18), all purchased from Santa Cruz Biotechnology, anti-Bik (Abgent), anti-caspase 9, and anti-caspase 3 (Cell Signaling Technology; New England Biolabs), anti-cytochrome c (7H8;2C12; BD Biosciences), and anti-Bid, anti-caspase 8, both purchased from Mo BI Tec. Secondary Abs for Western blot analysis were purchased from Sigma-Aldrich and Jackson ImmunoResearch Laboratories.

Histochemistry

Liver tissue was fixed in 4% formalin in PBS and subsequently embedded in paraffin. Sections were stained for H&E using a standard protocol and analyzed by light microscopy. In situ end labeling (ISEL) for detection of apoptotic cells was performed for paraffin-embedded tissue (25).

Real-time RT-PCR

Isolation of total RNA from cells was conducted using the Nucleo Spin RNA Purification kit (BD Clontech). To analyze altered gene expression, miRNA was transcribed into cDNA using SuperScript II RNase H− reverse transcriptase (Innogenix Life Technologies). Oligonucleotides for subsequent PCR were also obtained from Eurogentec Deutschland. Primer sequences were: β-actin: 5’-TGG AAT CCT GTG GCA TCC ATG AAA-3’ and 5’-TAA AAC GCA GCT CAG TAA CAC TGG C-3’; Bax: 5’-ACA TTG CTA CCA GAT AC-3’ and 5’-CTT CTT CTA GAT GAT GAG C-3’; A20: 5’-CAG GGT TCC AGA ACA AGA TGC ATC-3’ and 5’-CTC CAT ACA GAC TTT CCA-3’. Primers were designed using LightCycler rapid thermal cycler system (Roche Diagnostics) and the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). Reactions were performed in a 10-μl volume. To confirm amplification specificity, PCR products were subjected to a melting curve analysis and visualized by gel electrophoresis.

Cell culture and transfection

Hepa-1−6 cells were plated in 500 μl of RPMI 1640 containing 10% FCS (Innogenix Life Technologies) in 24-well plates at a number of 2 × 104 cells/well. Cells were allowed to adhere to culture plates overnight. Primary mouse hepatocytes were isolated by a modification of the two-step collagenase perfusion method of Seglen (43) from adult male BALB/c mice. Digestion step was performed with Liberase Blendzyme 3 recombinant collagenase (Roche Diagnostics) according to the manufacturer’s instructions. Cells were plated in 100 μl of RPMI 1640 (Innogenix Life Technologies) containing 10% basal medium supplement in 96-well tissue-culture plates (Greiner Bio-One) at a number of 4 × 103 cells/well and maintained at 37°C, 5% (v/v) CO2 and 40% ambient oxygen concentration (v/v). After cells were allowed to adhere to culture plates for 18 h, cell debris and dead cells were removed by scavenging with OptiMem I Medium (Innogenix Life Technologies). Transfections were performed using Lipofectamine 2000 (Innogenix Life Technologies) according to the manufacturer’s instructions. After 24 h, corresponding wells were prestimulated with recombinant murine TNF (40 ng/ml; Innogenix) for 8 h. Apoptosis was induced by administration of actinomycin D (75 nM) 30 min before administration of TNF (40 ng/ml).

Cloning and sequencing

A full-length expression clone of A20 (pA20) was obtained using the pCDNA3.1/V5-His-TOPO TA Expression kit (Innogenix Life Technologies) in combination with the Accuprime Taq Polymerase System (Invitrogen Life Technologies). Sequence analysis was done using the ABI PRISM 377 DNA sequencer (Applied Biosystems).

Luciferase assay

Hepa−1−6 cells were transfected in 24-well plates with 0.4 μg of luciferase reporter pB2LUC, containing 2 NF-xB-binding sites, or with 0.8 μg of pBaxLUC2.8, containing 2.8 kb of the upstream region of the bax start codon cloned in front of firefly luciferase. Twenty-four hours after transfection cells were incubated as described in the figure legends and harvested for extract preparation. Luciferase reporter activity was measured by a commercial assay (Luciferase Assay System; Promega).

Statistical analysis

The results were analyzed using the Student t test if two groups were compared and the Dunnett’s test if more groups were tested against a control group. If variances were inhomogeneous in the Student’s t test, the results were analyzed using the Welch test. All data in this study are expressed as a mean ± SEM. A value of p ≤ 0.05 was considered significant.
Results

**TNF treatment interferes with mitochondrial apoptosis and down-regulates Bax expression in vivo**

Administration of low doses of TNF, without transcriptional or translational inhibition, protects mice from liver damage subsequently induced by GalN/TNF administration (Fig. 1A). To elucidate the mechanism of TNF-induced protection, we measured downstream signals leading to liver damage in the GalN/TNF model and compared them to those in TNF-pretreated, protected mice.

Following induction of liver damage by GalN/TNF administration, we found activation of Bax-mediated mitochondrial apoptosis (Fig. 1B). Densitometric evaluation revealed significant activation of caspase 8, as measured by cleavage of the inactive form (median ± SEM: lines 1–3: 355.47 ± 16.97 relative units (RU) vs lines 4–6: 94.57 ± 61.43 RU; p ≤ 0.05) as well as significant cleavage of inactive p22 Bid to tBid (696.51 ± 14.09 RU vs 577.68 ± 21.98 RU; p ≤ 0.05). During activation, e.g., by tBid, Bax monomers assemble to oligomers in the mitochondrial membrane (26), which results in reduced amounts of the Bax monomer. Concomitantly, in the GalN/TNF model of liver damage, we detected significant activation of Bax, as measured by disappearance of the inactive monomer (650.12 ± 101.25 RU vs 219.59 ± 11.21 RU; p ≤ 0.05), representing a reduction of the Bax monomer by 66%.

In protected mice (Fig. 1C, lanes 4–6), we found significantly reduced activation of caspase 8 and Bid, as shown by significantly reduced cleavage of inactive caspase 8 as well as of p22 Bid to tBid. Subsequent events of mitochondrial apoptosis, i.e., release of cytochrome c and activation of caspase 9, as well as activation of the effector caspase 3 were also found to be attenuated in livers of TNF-pretreated animals (Fig. 1C). Interestingly, we detected almost complete disappearance (>90%) of the Bax monomer p21 in livers of TNF-pretreated mice (Fig. 1C), while we had expected...
this process to be interrupted or even reverted in case of protection, as seen for caspase 8 and Bid cleavage (compare Fig. 1, B and C). In contrast, Bax expression was significantly reduced (median ± SEM: lines 1–3: 526.9 ± 14.8 RU vs lines 4–6: 53.97 ± 22.59 RU; p ≤ 0.05) in livers of TNF-protected mice, which was obviously not due to activation, because subsequent events in mitochondrial apoptosis were missing (Fig. 1C). This prompted us to investigate the effect of TNF pretreatment alone on Bax expression. In cDNA arrays (17) as well as in real-time RT-PCR (Fig. 2A), we observed reduced expression of Bax while expression of other proapoptotic members of the Bcl2 family of proteins (Bak, Bad, Bik, or Bid) was not affected (Fig. 2C). Down-modulation of Bax by siRNA protected mice from apoptotic liver damage

To determine whether down-modulation of Bax expression, as observed following TNF treatment (Fig. 2, A and B), actually interferes with mitochondrial apoptosis, we designed siRNA directed specifically against murine Bax (siBax). In comparison to control siRNA (siControl), siBax down-modulated Bax expression in vivo by ~70% (Fig. 2C), while expression of other proapoptotic members of the Bcl2 family of proteins (Bak, Bad, Bik, or Bid) was not affected (Fig. 2C). Down-modulation of Bax by siRNA protected mice from GalN/TNF-induced liver damage (Fig. 3A) and reduced subsequent events in mitochondrial apoptosis, such as release of cytochrome c and activation of caspase 9 (Fig. 3B). The protective effect of siBax against apoptosis induced by GalN/TNF could also be demonstrated in liver sections stained with H&E (Fig. 3C, upper part) or by using ISEL to determine DNA damage (Fig. 3C, lower part).
These results indicate that indeed down-modulation of Bax expression, as observed following TNF pretreatment, is able to protect from liver damage.

Because liver damage induction by TNF exclusively occurs in the presence of hepatocyte transcriptional inhibition, there must be a TNF-inducible protein which in turn down-regulates Bax expression. This additional player in TNF tolerance could be A20, a protein which has been shown to be inducible in hepatocytes (27) and to protect from mitochondrial apoptosis (28). To investigate a possible connection between TNF, A20 induction and Bax down-modulation, we incubated Hepa1–6 cells in the presence of TNF for different periods of time and measured A20 as well as Bax expression by real-time RT-PCR. TNF induced the expression of A20 within 30 min for at least 3 h, while it decreased the expression of Bax from 3 h onward for at least 8 h (Fig. 4A). To investigate whether these processes were related, A20 was overexpressed in Hepa1–6 cells by transient transfection of an A20 expression clone for 12 or 24 h (Fig. 4B), resulting in significantly increased A20 expression (Fig. 4B, upper part) and decreased expression of Bax mRNA (Fig. 4B, lower part). These results indicate a connection between A20 expression and expressional down-regulation of Bax.

Down-regulation of A20 or overexpression of Bax interferes with TNF-induced protection in primary hepatocytes

To further investigate the hypothesis of a connection between A20-, Bax-, and TNF-induced protection, we isolated primary mouse hepatocytes and induced cellular damage by incubation with the transcriptional inhibitor Act.D in combination with TNF. Because liver damage induction by TNF exclusively occurs in the presence of hepatocyte transcriptional inhibition, there must be a TNF-inducible protein which in turn down-regulates Bax expression. This additional player in TNF tolerance could be A20, a protein which has been shown to be inducible in hepatocytes (27) and to protect from mitochondrial apoptosis (28). To investigate a possible connection between TNF, A20 induction and Bax down-modulation, we incubated Hepa1–6 cells in the presence of TNF for different periods of time and measured A20 as well as Bax expression by real-time RT-PCR. TNF induced the expression of A20 within 30 min for at least 3 h, while it decreased the expression of Bax from 3 h onward for at least 8 h (Fig. 4A). To investigate whether these processes were related, A20 was overexpressed in Hepa1–6 cells by transient transfection of an A20 expression clone for 12 or 24 h (Fig. 4B), resulting in significantly increased A20 expression (Fig. 4B, upper part) and decreased expression of Bax mRNA (Fig. 4B, lower part). These results indicate a connection between A20 expression and expressional down-regulation of Bax.

FIGURE 5. Overexpression of A20 or down-regulation of Bax interferes with TNF-induced protection in primary hepatocytes. Primary mouse hepatocytes were transfected with siRNA directed against GFP (siControl) or against Bax (siBax) alone or in combination with an expression clone for murine Bax (pBax) or an empty control vector. After 24 h, corresponding wells were stimulated with TNF (40 ng/ml) for 8 h. Apoptosis was induced by administration of Act.D (75 nM) 30 min before TNF (40 ng/ml). LDH release was measured after 18 h of incubation to calculate cytotoxicity. Data are expressed as the mean ± SEM (n = 20; *, p ≤ 0.05 for cells incubated with Act.D/TNF vs cells pretreated with TNF; n = 10–20; #, p ≤ 0.05 for cells pretreated with TNF vs cells pretreated with TNF after transfection with pBax and siA20, either alone or in combination).

These results indicate that indeed down-modulation of Bax expression, as observed following TNF pretreatment, is able to protect from liver damage.

FIGURE 6. A20 induces TNF tolerance by down-modulating Bax expression. C57BL/6 mice were pretreated with siRNA directed against GFP (siControl; lanes 1–6) or siRNA directed against A20 (siA20; lanes 7–9) as well as with HSA (lanes 1–3) or TNF (10 μg/kg) (lanes 4–9) for 12 h. Subsequently, liver damage was induced by application of GalN/TNF. Measurements were performed 6 h after GalN/TNF challenge. Liver damage was measured by (A) determination of ALT in plasma and (B) caspase-3 activity in liver homogenates by ELISA. Data are expressed as the mean ± SEM (n = 5; *, p ≤ 0.05 for HSA vs TNF pretreated mice; #, p ≤ 0.05 for siControl vs siA20 pretreated mice). C, A20 was detected by Western blot analysis 6 h after GalN/TNF challenge. D, Expression of caspase 8, Bid (22 kDa), and Bax (21 kDa) as well as release of cytochrome c and activation of caspase 9 were measured by Western blot analysis also 6 h after challenge. E, Signs of apoptosis were detected by H&E staining (upper part) and the ISEL staining method (lower part; apoptotic cells are stained in brown).
We found that pretreatment with TNF-protected hepatocytes from damage, while this protective effect was counteracted by either overexpressing Bax or interfering with A20 expression. This result was most obvious when both incubation regimens were combined (Fig. 5). Our results show that TNF-induced protection is also achievable in vitro and seems to depend on the presence of A20 as well as on the absence of Bax.

**A20 induces TNF tolerance by down-modulating Bax expression in vivo**

To investigate the contribution of A20 to TNF-induced tolerance and its connection to Bax expression in vivo, we administered siA20 or siControl to TNF-pretreated mice and subsequently measured GalN/TNF-treated liver damage. Down-modulation of A20 expression was ~90% efficient in vivo (Fig. 6C). A20 knockdown in TNF-pretreated mice restored their sensitivity toward GalN/TNF-induced apoptosis and liver damage (Fig. 6A) as manifested by activation of caspase 3 (Fig. 6B). A20 knockdown partially restored activation of caspase 8 and Bid, and almost completely restored Bax expression, release of cytochrome c and activation of caspase 9 (Fig. 6D). Restoration of apoptosis by siA20 in GalN/TNF-treated mice could also be observed in liver slices stained with H&E (Fig. 6E, upper part) as well as in liver slices where DNA damage was determined by ISEL staining (Fig. 6E, lower part). These experiments indicate that TNF-induced expression of A20 is responsible for down-regulation of Bax expression and also for reduced activation of residual Bax.

**TNF-induced expression of A20 results in decreased NF-κB activation and subsequently in reduced Bax expression**

In an attempt to explain how A20 might be able to interfere with Bax expression, we performed additional in vitro experiments. Because A20 is known to interfere with NF-κB activation (29, 30), we first investigated the impact of NF-κB inhibition on Bax expression. Real-time RT-PCR analysis revealed that NF-κB inhibition by gliotoxin (21) interfered with Bax expression starting at concentrations of 100 nM (Fig. 7A), while concentrations of gliotoxin lower than 1 μM were not toxic to the cells during 24 h of incubation time, as measured by release of lactate dehydrogenase (data not shown). The effect of gliotoxin incubation on NF-κB activity was confirmed in luciferase assays using a construct containing luciferase under the control of a promoter containing 3 B-binding sites (pB2Luc; Fig. 7B). Incubation with TNF (Fig. 7C) or with 100 nM gliotoxin (Fig. 7D) was able to interfere with the expression of a construct containing a full-length Bax promoter region fused to the luciferase gene (pBaxLuc2.8.8). Finally, expression of pBaxLuc2.8 was significantly lower in a cell line stably overexpressing A20 (Fig. 7E). These results indicate that NF-κB inhibition, as performed by an inhibitor or by overexpression of A20, is able to interfere with Bax promoter activity. Therefore, the mechanism of TNF-induced protection seems to involve induction of A20 expression, A20-mediated inhibition of NF-κB activation and, subsequently, reduced expression of the proapoptotic Bax gene.

**Discussion**

TNF, as a proinflammatory cytokine, plays a pivotal role in several severe human diseases such as rheumatoid arthritis or Crohn’s disease (31). TNF is also implicated in apoptotic liver damage, seen during viral hepatitis, inflammatory hepatitis, endotoxemia-induced liver failure, and ischemia/reperfusion-induced liver damage (2), and therefore promotes inflammation-related processes resulting in apoptotic and necrotic destruction of organs. Accordingly, therapy of the above-mentioned syndromes involves blockade of TNF action, either by application of anti-TNF Abs (infliximab) or soluble TNFR2 (etanercept) (31). However, in December 2004, the Food and Drug Administration revised the labeling of the anti-TNF therapeutic remicade to include a warning on severe hepatic reactions (www.fda.gov/medwatch/SAFETY/2004/Remicade), and a recent report implicates a connection between anti-TNF therapy and the induction of autoimmune diseases (6), suggesting that TNF exerts also beneficial effects. Moreover, it has been shown that TNF-preconditioning protects from ischemia-reperfusion injury (5), a finding which could improve the outcome of transplantations in patients. Unfortunately, preconditioning with TNF itself would bear a high potential of risk, because TNF is a proinflammatory cytokine and involved in apoptotic organ destruction (2). Therefore, we intended to provide knowledge on mechanisms and mediators of protection induced by TNF, to identify novel protective factors for future gene therapy that might have similar effects as TNF preconditioning, but circumvent its risks.
against Bax mRNA, we could in fact demonstrate that a lack of Bax itself, and not only a lack of Bax activation, can interfere with the onset of mitochondrial apoptosis. This result prompted us to investigate signaling pathways leading from TNF to reduction of Bax expression in vitro as well as in vivo.

TNF induces apoptotic liver damage in mice only in the presence of hepatocyte-specific transcriptional inhibition. Without such inhibition, injection of TNF alone protects mice from liver injury. Hence, there must be at least one TNF-inducible protein in hepatocytes that protects from detrimental TNF effects. We identified the NF-κB-inducible protein A20 (36) as the missing link. A20, a zinc finger protein originally discovered in endothelial cells (37), has been shown to protect from TNF-induced apoptosis in vitro (38) as well as in vivo (27), and to stabilize the mitochondrial membrane potential (28). As an immediate early gene, A20 is inducible by NF-κB-activating stimuli, such as TNF or LPS (36, 39), within a very short period of time, due to a constitutive association of the general transcription apparatus (40). In contrast, in a feedback loop, A20 inhibits the activation of NF-κB and other transcription factors (29, 30, 41). A20 is a hepatoprotective (27), TNF-inducible protein in hepatocytes and has been shown to protect cells, which are deficient in NF-κB activation, from TNF-induced apoptosis by disrupting the recruitment of the death domain-signaling molecules TRADD and receptor-interacting protein (RIP) to the receptor signaling complex (42). Our results show that TNF induces A20 expression before Bax down-modulation, and that A20 overexpression is able to interfere with TNF expression on transcriptional level. Using siRNA against A20 or Bax overexpression in vitro, we were able to reverse TNF-induced protection from cellular damage in primary hepatocytes. Moreover, we could demonstrate in vivo that interference with A20 expression restored Bax expression and subsequent events in mitochondrial apoptosis, including cytochrome c release and caspase 9 as well as caspase 3 activation. Concerning the mechanism by which A20 is able to interfere with Bax expression, and knowing that A20 inhibits NF-κB activation (29, 30), we investigated the dependence of Bax expression on NF-κB activation. Using an NF-κB inhibitor and a fusion construct of the Bax promoter and luciferase, we could indeed demonstrate that Bax expression is inducible by NF-κB. A20 also seems to be able to interfere with the activation of AP1 (29). Therefore, it might be possible that A20-induced interference with the activity of other transcription factors might also contribute to TNF-induced hepatoprotection.

Taken together, our results show that TNF-induced protection seems to involve a mechanism by which TNF induces the expression of A20 in hepatocytes, A20 down-modulates Bax expression by interference with, e.g., NF-κB activation, and reduced availability of Bax interferes with the onset of mitochondrial apoptosis and the ensuing apoptotic liver damage. In addition to this process TNF pretreatment interferes with activation of Bid, a prerequisite for activation of residual Bax protein. Cyt c: cytochrome c.

It has been shown that TNF pretreatment does not down-regulate the amount of TNFR1 on hepatocytes (16), the receptor which is necessary for induction of damage, as well as for induction of tolerance. Furthermore, it has been shown that neither IL-1, nor heme oxygenase-1, nor inducible NO synthase are mediators of TNF tolerance (16), although inducible NO synthase-derived NO contributes to endotoxin tolerance (32), which therefore is clearly distinguishable from TNF tolerance. Nevertheless, because under experimental conditions, e.g., in mouse models or in cell culture, TNF exerts its proapoptotic effects only in the presence of transcriptional inhibition, TNF tolerance seems to depend on the activation of yet unknown TNF-inducible cytoprotective proteins. To identify those proteins, we performed cDNA arrays on livers of TNF-treated mice (17). We found that in fact numerous potentially antiapoptotic genes were induced, but, more intriguing, TNF also reduced the expression of proapoptotic members of the Bcl2 family at the transcriptional level. Among these genes we identified Bax, which is a key factor in the onset of mitochondrial apoptosis. In the initial process of mitochondrial apoptosis, TNF/TNFR1 interaction results in the recruitment of TNFR-associated death domain-containing protein (TRADD) leading to NF-κB activation, or to an interaction with Fas-associated death domain-containing protein (FADD) resulting in activation of caspase 8 (33). Activation of caspase 8 promotes cleavage of Bid to tBid, which subsequently is able to activate Bax (18), while NF-κB activation is able to induce cytoprotective proteins (Refs. 34 and 35; Fig. 8). Indeed, we found that Bid is activated in our mouse model, and that TNF pretreatment seems to interfere with its expression, while it does not influence its expression. Upon activation, cytosolic Bax disappears from the cytoplasm, integrates into the mitochondrial membrane, oligomerizes, forms pores, and facilitates the release of the pro-apoptotic activator cytochrome c from the mitochondrion (19), resulting in subsequent activation of the effector caspase 3 (Ref. 20; Fig. 8). We observed that TNF pretreatment, besides interference with events upstream events in Bax activation, was very efficient in interfering with Bax expression. Using siRNA directed specifically...
there are still many obstacles to be circumvented before this technology will be useable in human therapy, which include reduction of siRNA amounts necessary for efficient knockdown and improvement of cell specificity.

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Disclosures
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