Hepatocyte-specific inhibition of NF-κB leads to apoptosis after TNF treatment, but not after partial hepatectomy

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One of the earliest TNF-dependent events to occur during liver regeneration is the activation of the transcription factor NF-κB through TNF receptor type 1. NF-κB activation in the liver can have both antiapoptotic and proliferative effects, but it is unclear which liver cell types, hepatocytes or nonparenchymal cells (NPCs), contribute to these effects. To specifically evaluate the role of hepatocyte NF-κB, we created GLVP/ΔN-IκBα transgenic mice, in which expression of a deletion mutant of IκBα (ΔN-IκBα) was induced in hepatocytes after injection of mifepristone. In control mice, injection of 25 μg/kg TNF caused NF-κB nuclear translocation in virtually all hepatocytes by 30 minutes and no detectable apoptosis, while in mice expressing ΔN-IκBα, NF-κB nuclear translocation was blocked in 45% of hepatocytes, leading to apoptosis 4 hours after TNF injection. In contrast, expression of ΔN-IκBα in hepatocytes during the first several hours after partial hepatectomy did not lead to apoptosis or decreased proliferation. As NF-κB activation was not inhibited in liver NPCs, it is likely that these cells are responsible for mediating the proliferative and antiapoptotic effects of NF-κB during liver regeneration.

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Introduction

The transcription factor NF-κB has been implicated in both hepatocyte proliferation and apoptosis. Mice deficient in the p65 subunit of NF-κB die during gestation from hepatocyte apoptosis (1). Inhibition of NF-κB in hepatocyte cell lines blocked TNF-induced proliferation and sensitized these cells to apoptosis (2, 3). Furthermore, while inactive in adult, quiescent liver, NF-κB is rapidly activated after partial hepatectomy (PH), a surgical procedure that stimulates a process of compensatory proliferation known as liver regeneration (4, 5).

NF-κB can be activated by multiple inflammatory stimuli, including cytokines such as TNF and IL-1, as well as bacterial endotoxin. These agents activate NF-κB by signaling kinases to phosphorylate the inhibitor of NF-κB, IκBα, which targets it for ubiquitination and subsequent degradation by the proteasome (6). Degradation of IκBα releases NF-κB to translocate from the cytosol to the nucleus, where it can activate transcription of genes containing appropriate NF-κB binding sites. NF-κB transcription can be inhibited by overexpression of a nondegradable IκBα mutant that no longer contains N-terminal phosphorylation sites. Several groups have used this "super-repressor" IκBα to show that inhibition of NF-κB sensitizes multiple cell types, including hepatocytes, to apoptosis (2, 7–10).

TNF signaling through its type 1 receptor (TNFR1) plays a particularly important role in NF-κB activation in the liver. The lethal hepatocyte apoptosis observed in p65 knockout mice can be rescued by crossing these mice into a TNF or TNFR1-null background (11, 12). TNFR1/p65 double-knockout mice die shortly after birth due to massive acute inflammation of the liver. While TNFR1 knockout mice develop normally, they are severely impaired for liver regeneration and are deficient in activation of NF-κB after PH as well as downstream events such as IL-6 upregulation and STAT3 activation (13). Using a hepatocyte progenitor cell line, we showed that inhibition of NF-κB directly blocked TNF-mediated increases in IL-6 and STAT3 (3). However, other work has suggested that in vivo it is the liver nonparenchymal cells (NPCs) (i.e., resident macrophages, or Kupffer cells) that are responsible for IL-6 release (14, 15). Thus the cell-specific role of NF-κB in IL-6 production and hepatocyte proliferation during liver regeneration has yet to be clarified.

NF-κB can be activated in both hepatocytes and NPCs during liver regeneration (4, 5). Iimuro et al. (9) found increased hepatocyte apoptosis in the regenerating liver of rats injected before PH with an adenoviral vector containing the super-repressor IκBα. However, the viral vector by itself also caused increased TNF

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Nonstandard abbreviations used: Partial hepatectomy (PH); TNF receptor type 1 (TNFR1); nonparenchymal cells (NPCs); bromodeoxyuridine (BrdU); Tris-buffered saline/Tween 20 (TBST); inducible nitric oxide synthase (iNOS); electrophoretic mobility shift assay (EMSA).

levels, DNA synthesis, and apoptosis in the liver before PH. In addition, adenoviral vectors can cause hepatic macrophages to release IL-6 (16). Thus the results obtained by Iimuro et al. may not accurately reflect the physiologic role of NF-κB signaling in hepatocytes during liver regeneration.

To specifically evaluate the role of NF-κB in hepatocytes, we used a liver-specific inducible transgenic mouse system (17) to overexpress a nonphosphorylatable N-terminal deletion mutant of IκBα (ΔN-IκBα). Using this system, we found that specific expression of ΔN-IκBα in hepatocytes prior to TNF injection caused hepatocyte apoptosis but did not affect liver regeneration or cause hepatocyte apoptosis when expressed prior to PH. Additionally, we demonstrate that TNF-induced hepatocyte apoptosis caused by inhibition of hepatocyte NF-κB or sensitization with D-galactosamine occurs only at high doses of TNF, suggesting that the mouse liver has multiple protective mechanisms that prevent hepatocyte apoptosis.

Methods

Transgenic mice and animal procedures. Mice expressing the chimeric transcriptional activator GLVP under the control of the liver-specific transthyretin promoter were previously described (17). For GAL4/*ΔN-IκBα* transgenic mice, a PCR-generated fragment encoding an N-terminal–deleted IκBα gene with a FLAG epitope tag (18) was subcloned into a plasmid containing the GAL4 promoter, 17x4-tk-CAT, replacing CAT with ΔN-IκBα. The resulting plasmid was sequenced and linearized prior to injection into fertilized 1-day old eggs of C57BL6/C3H origin. Injected eggs were implanted into CD-1 females, and the resulting progeny were screened for the presence of the transgene by PCR analysis of tail DNA. Mice carrying the GAL4/*ΔN-IκBα* transgene were crossed to C57BL6 mice (The Jackson Laboratory, Bar Harbor, Maine, USA) to obtain colony founders. Hemizygous GAL4/Δ*N-IκBα* transgenic mice were then crossed to homozygous transthyretin/*GLVP* transgenic mice to obtain both single-transgenic and double-transgenic littermates. Offspring were analyzed for the presence of both transgenes by PCR of tail DNA. For all experiments, 8- to 12-week-old male mice were used. Animals were maintained and treated according to protocols approved by the University of Washington Animal Care Committee.

Three hours prior to TNF injection or PH, mice were injected intraperitoneally with 5 mg/kg mifepristone (Sigma-Aldrich, St. Louis, Missouri, USA) dissolved in sesame oil. For one experiment, mice were injected with mifepristone at 12-hour intervals to maintain expression of the transgene throughout liver regeneration. Two-thirds PH was performed as previously described (19). Mice were anesthetized with Metofane and killed after hepatectomy at the times indicated in the figures. To measure DNA replication, mice were injected with 50 μg/g bromodeoxyuridine (BrdU; Roche Diagnostics Inc., Indianapolis, Indiana, USA) 2 hours prior to killing. Liver samples were fixed overnight in buffered formalin or methyl Carnoy's fluid for immunohistochemistry or were frozen immediately in liquid nitrogen. Nuclear extracts were prepared from fresh tissue as previously described (13). Serum samples were collected by cardiac puncture for use in IL-6 ELISA (BD Pharmingen, San Diego, California, USA) or AST/ALT measurements (Sigma-Aldrich). For experiments involving TNF, mice were injected with 5 μg/kg or 25 μg/kg of recombinant murine TNF (R&D Systems Inc., Minneapolis, Minnesota, USA) dissolved in saline, 3 hours after mifepristone injection or immediately after injection of 0.9 g/kg D-galactosamine (Calbiochem-Novabiochem Corp., La Jolla, California, USA).

Western blot analysis and electrophoretic mobility shift assay. Protein lysates were prepared as previously described (20). Fifty-microgram samples were separated by 15% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Equivalent loading was confirmed by Ponceau S staining and membranes were blocked overnight in 5% milk dissolved in 0.1% Tween-20/Tris-buffered saline (TBST) at 4°C. IκBα was detected with C-terminal–specific rabbit anti-IκBα (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) at 1:5,000 dilution in 0.5% milk/TBST or with an anti-FLAG mouse monoclonal antibody (M5; Sigma-Aldrich) at 1:300 in TBS. Active caspase-3 antibody (BD Pharmingen) and inducible nitric oxide synthase (iNOS) antibody (Sigma-Aldrich) were each used at 1:2,000 dilution. Following a 1-hour incubation with primary antibodies, blots were washed with TBST, probed with specific secondary antibodies (Santa Cruz Biotechnology Inc.), washed again with TBST, and incubated with chemiluminescent substrate (NEN Life Science Products Inc., Boston, Massachusetts, USA). For electrophoretic mobility shift assay (EMSA), 5 μg of nuclear extract protein was incubated with 4×10^4 cpm ^{32}P-labeled NF-κB or STAT3 consensus oligonucleotides for 30 minutes in binding buffer, as previously described (5). Samples were resolved on 5% 1× TGE acrylamide gels and subsequently analyzed by autoradiography.

Immunohistochemistry. Fixed liver sections were stained with an antibody to BrdU (DAKO Corp., Carpinteria, California, USA) as previously described (21). For detection of the p65/RelA subunit of NF-κB, formalin-fixed liver sections were rehydrated, microwaved for antigen retrieval in 10 mM sodium citrate buffer, and incubated with a goat anti-p65 antibody (SC-372-G; Santa Cruz Biotechnology Inc.) diluted 1:100 in 5% normal rabbit serum. Slides were treated with the goat VECTASTAIN Elite ABC kit (Vector Laboratories Inc., Burlingame, California, USA) and diaminobenzidine (Sigma-Aldrich), followed by counterstaining with hematoxylin and eosin.

Caspase and TUNEL assays. One hundred micrograms of liver protein lysates were incubated with a fluorogenic caspase-3 substrate, DEVD-AMC (Alexis Corp.,

San Diego, California, USA), for 1 hour. Fluorescence was quantitated as previously described (22) using a fluorescent plate reader (Packard Instrument Co., Meriden, Connecticut, USA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. For the TUNEL assay, apoptotic nuclei were detected using the POD In Situ Cell Death Detection kit (Roche Diagnostics Inc.) according to the manufacturer's instructions. Positive nuclei were counted in 30 fields (×400) for each slide.

Northern blot analysis. RNA was prepared from liver tissue using RNeasy (QIAGEN Inc., Valencia, California, USA). Separation and transfer of RNA was performed as previously described (23). Bcl-x_L probe was kindly provided by David Hockenbery (Fred Hutchinson Cancer Research Center, Seattle, Washington, USA), and cyclophilin probe was purchased from Ambion Inc. (Austin, Texas, USA).

Statistical analyses. Statistical analyses were performed using either GraphPad Prism version 2.0 (GraphPad Software Inc., San Diego, California, USA) or Microsoft Excel (Microsoft Corp., Redmond, Washington, USA). Results are presented as mean ± SEM. The one-tailed Student *t* test was used to calculate probability values; $P < 0.05$ was considered significant.

Results

Creation of a hepatocyte-specific, inducible ΔN-IκBα transgenic mouse. We used the GLVP transgenic mouse system (17) to inducibly express ΔN-IκBα in hepatocytes. GLVP is a chimeric transcriptional activator that contains a mutated progesterone ligand–binding domain fused to a yeast GAL4 DNA–binding domain and a viral transcriptional activator (24). GLVP cannot bind to endogenous promoter regions, and requires the presence of a progesterone antagonist, mifepristone, to bind to the GAL4 promoter and activate transcription of the downstream gene (Figure 1a). To establish a hepatocyte-specific inducible mouse system, GLVP was placed under control of the constitutive liver-specific promoter transthyretin (17). We crossed homozygous transthyretin/*GLVP* mice to hemizygous GAL4/*ΔN-IκBα* transgenic mice to obtain both single-transgenic GLVP and double-transgenic *GLVP*/*ΔN-IκBα* littermates. The level and duration of induced expression of the ΔN-IκBα protein in double-transgenic mice was evaluated by Western blot analysis of liver protein lysates. To determine whether ΔN-IκBα was expressed in double-transgenic mice in response to mifepristone treatment, these mice were injected with vehicle alone (sesame oil) or with 0.5, 1, or 5 mg/kg mifepristone (Figure 1b, top panel). Three hours after injection, mice were killed and liver lysates were prepared. Western blot analysis was performed using either a C-terminal–specific antibody to IκBα or an antibody to the FLAG epitope. No expression of ΔN-IκBα was observed in double-transgenic mice injected with sesame oil, but expression of a band migrating below the endogenous IκBα that corresponded to the transgenic protein increased with the dose of mifepristone. This band was also detected using an antibody to the FLAG epitope tag incorporated into the ΔN-IκBα protein (Figure 1b, bottom panel). As expected, single-transgenic mice did not express the ΔN-IκBα protein (data not shown). Analysis of the duration of ΔN-IκBα protein expression showed high expression between 3 hours and 6 hours after injection with 5 mg/kg mifepristone, decreasing substantially by 24 hours after injection (Figure 1c). The tissue-specificity of ΔN-IκBα expression was determined by Western blot analysis of multiple tissue protein lysates prepared from a double-transgenic mouse injected with 5 mg/kg mifepristone (Figure 1d). ΔN-IκBα protein was detected only in the liver.

Induced expression of ΔN-IκBα inhibits TNF-induced NF-κB nuclear translocation in hepatocytes. Next we wished

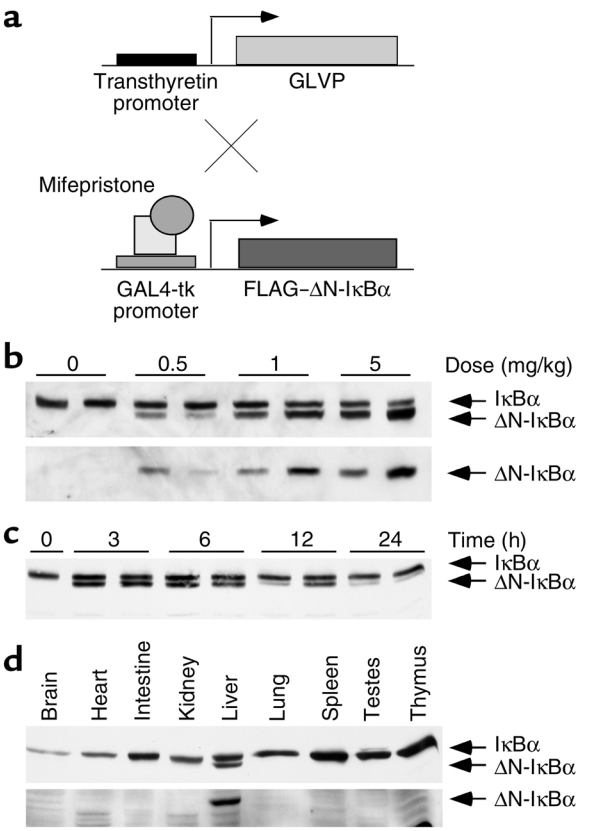

Figure 1
Induced expression of ΔN-IκBα in double-transgenic mice injected with mifepristone. (**a**) Schematic of the inducible transgenic mouse system used. (**b**) Mifepristone dose–dependent expression. Mice were killed 3 hours after intraperitoneal injection of the indicated dose of mifepristone dissolved in sesame oil. Western blot analysis of liver protein lysates was performed using an antibody to either the C terminus of IκBα (top panel) or to the FLAG epitope (bottom panel). Lanes represent individual animals. (**c**) Timing of induced expression. Mice were killed at the indicated times after injection of 5 mg/kg mifepristone. (**d**) Liver-specific expression of ΔN-IκBα. Organs were harvested from a mouse killed 3 hours after injection with 5 mg/kg mifepristone. Western blot analysis was performed using an antibody to IκBα (top panel) or FLAG (bottom panel).

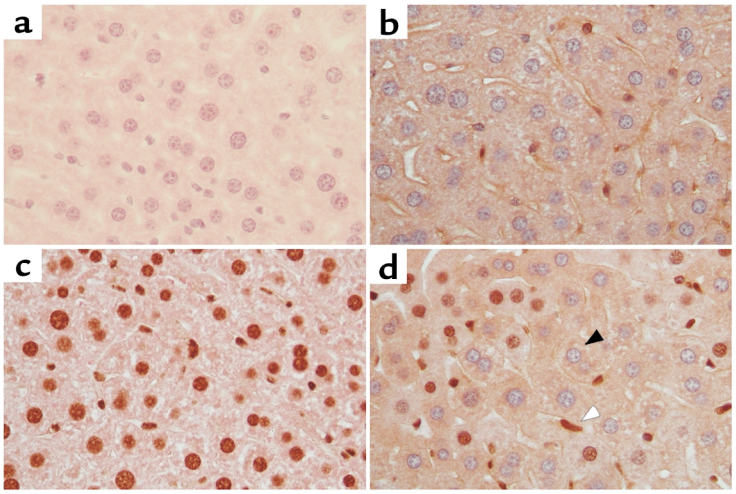

Figure 2
Inhibition of TNF-induced p65 nuclear translocation in hepatocytes of double-transgenic mice expressing ΔN-IκBα. Liver sections were stained with an antibody to the p65 subunit of NF-κB as described in Methods. (**a**) Negative control: no primary staining of a single-transgenic mouse killed 3 hours after injection with 5 mg/kg mifepristone. (**b**) Same tissue stained with p65 antibody. (**c** and **d**) p65 staining of a mifepristone-treated single-transgenic mouse (**c**) or double-transgenic mouse (**d**) killed 30 minutes after injection of 25 μg/kg TNF. Open arrow indicates an NPC with nuclear p65 staining. Filled arrow indicates a hepatocyte in which p65 nuclear translocation has been blocked. Original magnification, ×400.

to determine the proportion and type of liver cells in which activation of NF-κB was inhibited by induced expression of ΔN-IκBα. As we were unable to develop a technique to directly detect ΔN-IκBα protein by immunohistochemistry for the FLAG epitope, we analyzed the extent to which induced expression of ΔN-IκBα inhibited TNF-induced activation of NF-κB by staining liver sections with an antibody to the p65/RelA subunit of NF-κB. In both single- and double-transgenic mice injected with 5 mg/kg mifepristone, p65 protein was localized in the cytoplasm of hepatocytes (Figure 2b and Table 1). A small number of NPCs had nuclear accumulation of p65 (approximately 10 NPCs per 100 hepatocytes). Injection of single-transgenic mice with 25 μg/kg TNF, a potent activator of NF-κB (25), led to rapid nuclear accumulation of p65 by 30 minutes after injection in 100% of hepatocytes (Figure 2c and Table 1). Additionally, a fourfold increase in nuclear staining in NPCs was observed. In double-transgenic mice expressing ΔN-IκBα, TNF-induced nuclear translocation of p65 in hepatocytes was significantly inhibited, with only 55% ± 9% of hepatocyte nuclei staining positive for p65 (Figure 2d and Table 1). However, p65 nuclear translocation was not inhibited in liver NPCs of double-transgenic mice (Table 1), demonstrating the hepatocyte-specific expression of ΔN-IκBα.

Inhibition of hepatocyte NF-κB allows TNF to activate apoptosis in mouse liver. While inhibition of NF-κB has been shown to sensitize multiple cell types (including hepatocytes) to TNF-induced apoptosis in vitro, the effect of inhibiting NF-κB in hepatocytes in vivo during TNF treatment has not been previously determined (2, 7, 8). TNF does not normally cause hepatocyte apoptosis in vivo unless mice are coinjected with an agent that inhibits transcription, such as D-galactosamine or actinomycin D (26). To determine the effect of the inhibition of hepatocyte NF-κB activation during TNF treatment in vivo, single- and double-transgenic mice were injected with 25 μg/kg TNF 3 hours after injection with 5 mg/kg mifepristone, and were killed 4 hours later. Liver protein lysates were analyzed for caspase-3 activity by both a fluorogenic caspase assay (Figure 3a, graph) and Western blotting for active caspase-3 (Figure 3a, inset). In single-transgenic mice treated with mifepristone and TNF, no increase in caspase activity was detected compared with mice injected with sesame oil and saline (vehicle controls) or with double-transgenic mice injected with sesame oil and TNF. In contrast, a 50-fold increase in caspase-3 activity was detected in liver lysates from double-transgenic mice injected with mifepristone (and thus expressing ΔN-IκBα) and TNF. This level of caspase activity was comparable to that measured in C57BL6 mice injected with TNF and D-galactosamine, which causes fulminant hepatitis and death of mice by 6–8 hours after injection (27). Apoptosis was also confirmed histologically by use of the TUNEL assay that detects fragmented DNA in apoptotic cells (Figure 3b). In four double-transgenic mice injected with mifepristone and TNF, between 2% and 11% of hepatocyte nuclei were TUNEL-positive, a similar range to that observed in mice injected with TNF and D-galactosamine (not shown). No TUNEL-positive cells were detected in single-transgenic mice injected with

Table 1
Inhibition of p65 nuclear translocation in hepatocytes of double-transgenic mice expressing ΔN-IκBα

	Untreated		TNF	
Genotype	Hepatocytes	NPCs	Hepatocytes	NPCs
G	0 ± 0	10 ± 3	100 ± 0	43 ± 3
GN	0 ± 0	10 ± 2	55 ± 9[A]	47 ± 5[B]

All mice were injected with 5 mg/kg mifepristone. Mice in the untreated group were killed 3 hours later; mice injected with 25 μg/kg TNF were killed 30 minutes later. Three to four mice were analyzed per group. Liver sections were stained with an antibody to p65 as described in Methods. For hepatocytes, numbers represent percentage of total hepatocyte nuclei staining positive for p65 protein. For NPCs, numbers indicate p65-positive nuclei per 100 hepatocytes counted. [A]$P < 0.001$ for p65-positive hepatocytes counted in TNF-treated double-transgenic mice compared with TNF-treated single-transgenic mice. [B]$P = 0.1424$ for p65-positive NPCs in TNF-treated double-transgenic mice compared with TNF-treated single-transgenic mice.

mifepristone and TNF. To confirm that apoptosis correlated with a decrease in NF-κB transcription factor activity, liver protein lysates were analyzed for expression of iNOS, a protein that has been implicated in mediating NF-κB–dependent antiapoptotic effects in hepatocytes (28). iNOS expression was detected in livers of single-transgenic mice injected with mifepristone and TNF, but was substantially decreased in similarly treated double-transgenic mice (Figure 3c).

While double-transgenic mice injected with mifepristone and TNF had high levels of caspase activity and apoptotic hepatocytes, no mortality was observed, in contrast to that observed in response to TNF and D-galactosamine. We postulated that loss of hepatocytes would lead to compensatory proliferation of remaining hepatocytes. Indeed, we observed hepatocyte DNA replication, as measured by BrdU incorporation, at 24–96 hours after injection of TNF in mifepristone-treated double-transgenic mice, but not in single-transgenic mice (Figure 3d), indicating that apoptotic cell death was sufficient to stimulate a proliferative response in the remaining hepatocytes.

Expression of ΔN-IκBα in hepatocytes does not lead to apoptosis after PH. A previous study by Iimuro et al. showed that infection of rats with an adenovirus containing a super-repressor IκBα prior to PH did not interfere with hepatocyte DNA replication but caused increased hepatocyte apoptosis at 24 hours after PH (9). We analyzed livers from mifepristone-injected single- and double-transgenic mice at 4, 8, and 24 hours after PH. Although similar levels of ΔN-IκBα protein were induced in double-transgenic mice after PH or TNF injection (Figure 4a), we did not observe any evidence of hepatocyte apoptosis after hepatectomy, either by measurements of caspase activity (Figure 4b) or histological evaluation (not shown). Interestingly, we observed almost complete degradation of the endogenous IκBα at 30 minutes after TNF treatment but not after PH (Figure 4a). Since the adenoviral vector used by Iimuro et al. caused an increase in levels of TNF mRNA in the liver compared with levels in untreated animals, the hepatocyte apoptosis observed in those experiments may have been due to the combination of increased TNF and inhibition of NF-κB. Indeed, this appears to be the case since the injection of double-transgenic mice with TNF 30 minutes prior to PH did lead to hepatocyte apoptosis as measured by caspase-3 activity (Figure 4b). Additionally, we observed increased expression of the antiapoptotic gene *Bcl-x*$_L$ after PH as previously reported (29), but not after TNF injection (Figure 4c). Since the increased expression was similar between single- and double-transgenic mice, this indi-

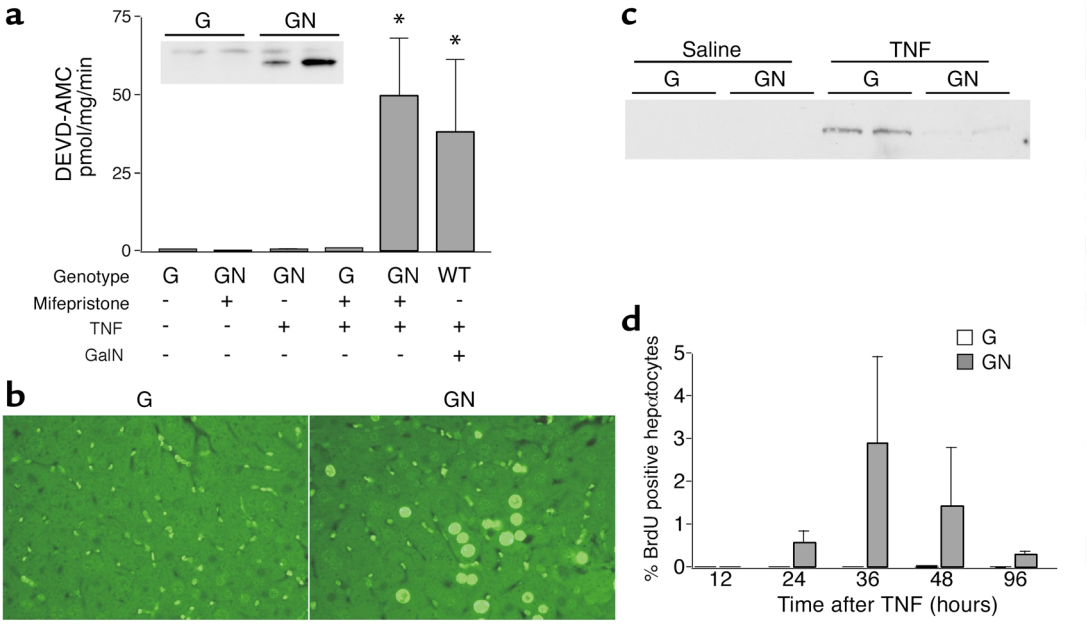

Figure 3
TNF activates hepatocyte apoptosis in double-transgenic mice expressing ΔN-IκBα. (**a**) Caspase-3 activity. Single-transgenic (G) or double-transgenic (GN) mice were injected with 25 μg/kg TNF or saline 3 hours after injection with mifepristone or sesame oil. For comparison, C57BL6 wild-type (WT) mice were coinjected with TNF and D-galactosamine (GalN). Mice were killed 4 hours after TNF injection and liver protein lysates were used to measure caspase-3 activity by incubation with the fluorogenic substrate DEVD-AMC. Data represent average (± SEM) caspase-3 activity measured in each group. Three to five mice were used per group. *$P < 0.05$ compared with vehicle control. Inset: active caspase-3 Western blot analysis of mifepristone- and TNF-treated mice. (**b**) TUNEL assay. Liver sections from a single-transgenic (left) and a double-transgenic (right) mouse treated with mifepristone and TNF were incubated with FITC-dUTP and terminal deoxynucleotidyl transferase. Original magnification, ×400. (**c**) iNOS Western blot. (**d**) DNA synthesis. Mice were injected with BrdU prior to sacrifice at the indicated times after treatment with mifepristone and TNF. Liver sections were stained with an antibody to BrdU as described in Methods, and positive nuclei were scored for 30 fields (×400) per section. Data represent average (± SEM) percentage of BrdU-positive hepatocyte nuclei per group. Three mice were used per group.

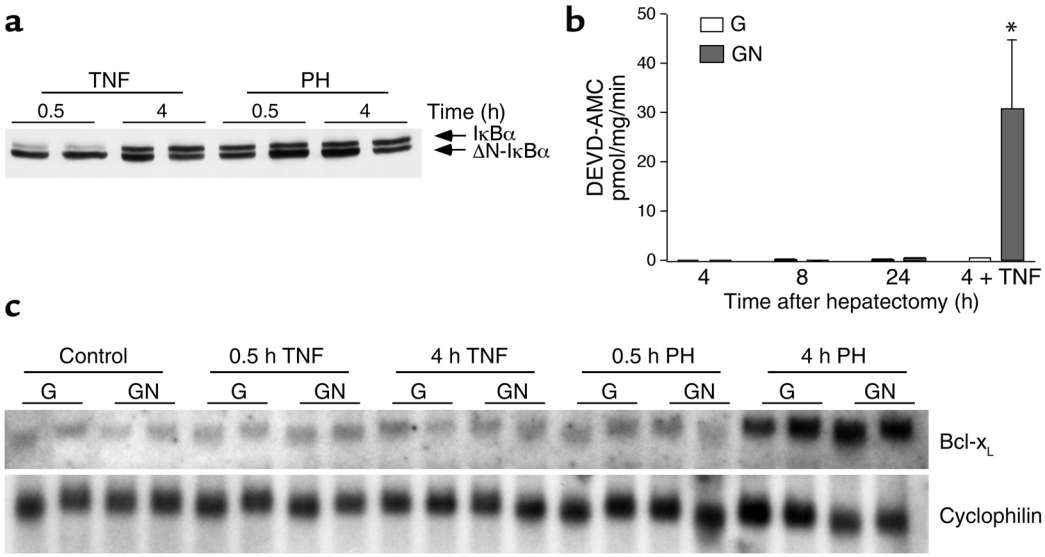

Figure 4

PH does not cause hepatocyte apoptosis in double-transgenic mice expressing ΔN-IκBα. (**a**) IκBα Western blot. Liver lysates were prepared from mifepristone-treated double-transgenic mice killed 30 minutes or 4 hours after injection of 25 μg/kg TNF (left lanes) or PH (right lanes). (**b**) Caspase-3 activity measured using substrate DEVD-AMC. Liver protein lysates were prepared from mifepristone-treated single-transgenic and double-transgenic mice killed at the indicated times after PH. The last column (4 + TNF) represents mice injected with 25 μg/kg TNF 30 minutes prior to PH and killed 4 hours after surgery. Data represent average (± SEM) caspase activity measured in each group. Three mice were used per group. $*P < 0.05$ for double-transgenic compared with single-transgenic mice. (**c**) Bcl-x_L Northern blot (top panel) and loading control cyclophilin (bottom panel).

cates that antiapoptotic pathways that are not dependent on NF-κB are activated during liver regeneration.

Effect of ΔN-IκBα expression on NF-κB activation after hepatectomy. Our previous studies and those from others showed that NF-κB activation increased after PH, beginning at 30 minutes and peaking at 1–2 hours after PH (4, 5). To determine the effect of ΔN-IκBα expression in hepatocytes on NF-κB activation after PH, mifepristone-treated single- and double-transgenic mice were killed at 30 minutes, 1 hour, and 2 hours after the operation. Nuclear extracts were prepared from the remaining liver and used for EMSA of NF-κB DNA binding activity. In single-transgenic mice, NF-κB binding activity was high at 1 hour and 2 hours after PH (Figure 5a). The binding activity contained the p65/p50 NF-κB heterodimer, as verified by supershift of the band with p65 and p50 antibodies (not shown). This band was completely absent in nuclear extracts of double-transgenic mice at all timepoints, suggesting that there was a complete inhibition of NF-κB binding in the livers of these animals. However, such a result was not to be expected, because TNF-induced p65 translocation was blocked in approximately half of hepatocytes and not at all in NPCs. Since the ΔN-IκBa protein can translocate to the nucleus (30), we investigated whether the presence of this protein in the nuclear extracts might affect NF-κB probe binding in the EMSA. We detected ΔN-IκBα protein, as well as low levels of endogenous IκBα, in nuclear extracts of double-transgenic mice (Figure 5b). To determine whether the presence of ΔN-IκBα protein in the nuclear extract could inhibit NF-κB probe binding, we coincubated liver nuclear extracts from single- and double-transgenic mice killed at 2 hours after PH (Figure 5c). Coincubation of these extracts resulted in diminished NF-κB binding activity compared with that seen in the single-transgenic extract alone. Thus, it is difficult to obtain a precise measurement of the extent of NF-κB binding activity in double-transgenic mice using EMSA. To determine the extent to which induced expression of ΔN-IκBα blocked nuclear translocation of the p65 subunit of NF-κB in hepatocytes of double-transgenic mice after PH, liver sections of mifepristone-treated double-transgenic mice killed without PH or 2 hours after PH were stained with a p65 antibody (Figure 5d). We found that while no NF-κB nuclear translocation was detectable in hepatocytes, a fourfold increase in NF-κB nuclear translocation was observed in NPCs (similar to that observed in single-transgenic mice, data not shown).

Induced expression of ΔN-IκBα in hepatocytes does not affect hepatocyte proliferation or gain of tissue mass after PH. Inhibition of NF-κB in hepatocyte cell lines altered the ability of TNF to stimulate proliferation of these cells, switching the stimulus from a proliferative to an apoptotic response (2, 3). We analyzed the effect of inhibiting hepatocyte NF-κB on hepatocyte DNA replication during liver regeneration by inducing expression of ΔN-IκBα prior to PH. Mifepristone-injected single- and double-transgenic mice received an injection of BrdU prior to sacrifice at 24–48 hours after PH (Figure 6a). No significant differences were observed in hepa-

tocyte BrdU labeling in double-transgenic mice compared with single-transgenic mice at any timepoint analyzed. The number of replicating hepatocytes observed in these mice was similar to that of non–mifepristone-treated mice after PH (data not shown). We also measured the number of cells undergoing mitosis at 48 hours after PH in mifepristone-treated single- and double-transgenic mice, and observed no differences between these two groups (Figure 6b). Finally, the overall ability of the liver to regenerate was determined by measuring the liver/body weight ratio of single- and double-transgenic mice 2 weeks after PH. Both groups showed a similar restoration of liver mass (Figure 6c).

As expression of the ΔN-IκBα protein was short-lived (decreasing by 12 hours after mifepristone injection, Figure 1b) it remained possible that NF-κB activation at a later time during liver regeneration was responsible for driving hepatocyte proliferation. To determine the effect of sustained NF-κB inhibition, mice were injected with mifepristone at 12-hour intervals during the first 48 hours after PH. No differences were observed in BrdU incorporation between single- and double-transgenic mice (Figure 6d, graph), despite high expression of ΔN-IκBα in double-transgenic mice (Figure 6d, inset).

Induced expression of ΔN-IκBα in hepatocytes does not affect IL-6 production or STAT3 activation after PH. Inhibition of NF-κB in cultured liver LE6 cells blocks upregulation of IL-6 by TNF (3). As IL-6 is required for normal liver regeneration after PH (31), we wished to determine whether inhibition of NF-κB in hepatocytes in vivo would diminish IL-6 production after PH. Single- and double-transgenic mice were injected with 5 mg/kg mifepristone 3 hours prior to PH and were killed at 0, 0.5, 1, and 2 hours after hepatectomy to obtain serum for IL-6 ELISA. No significant differences were observed in IL-6 increases after PH in double-transgenic mice expressing ΔN-IκBα compared with nonexpressing single-transgenic littermates (Figure 6e). Additionally, we analyzed STAT3 binding activity in nuclear extracts prepared from liver tissue of these mice and found similar increases in STAT3 binding at 2 hours after PH in single- and double-transgenic mice (Figure 6f). These data demonstrate that activation of NF-κB in the liver NPCs (rather than hepatocytes) is likely responsible for IL-6 production and subsequent STAT3 activation after PH.

Activation of NF-κB occurs at a lower dose of TNF than that required to activate apoptosis. Activation of NF-κB during liver regeneration is dependent on TNF signaling through its type 1 receptor (13). As apoptosis was observed in mice expressing ΔN-IκBα after injection with 25 μg/kg TNF but not after PH, we compared the magnitude of NF-κB activation by these treatments. We prepared nuclear protein from livers of single-transgenic mice at 30 minutes and 2 hours after injection of 25 μg/kg TNF or after PH. EMSA of these nuclear extracts showed much higher NF-κB binding activity in mice treated with TNF than after PH (Figure 7a). Next, we analyzed the effect of varying doses of TNF on both NF-κB activation and apoptosis. C57BL6 mice were injected with saline or with 5 or 25 μg/kg TNF and were killed 30 minutes later for analysis of NF-κB binding activity. Liver sections were stained with an antibody to p65. Similar to the results shown in Figure 2, p65 nuclear translocation occurred in 100% of hepatocytes after injection with either dose of TNF (data not shown). EMSA analysis of nuclear protein prepared from livers of these mice showed similar increases in NF-κB binding activity at both 5

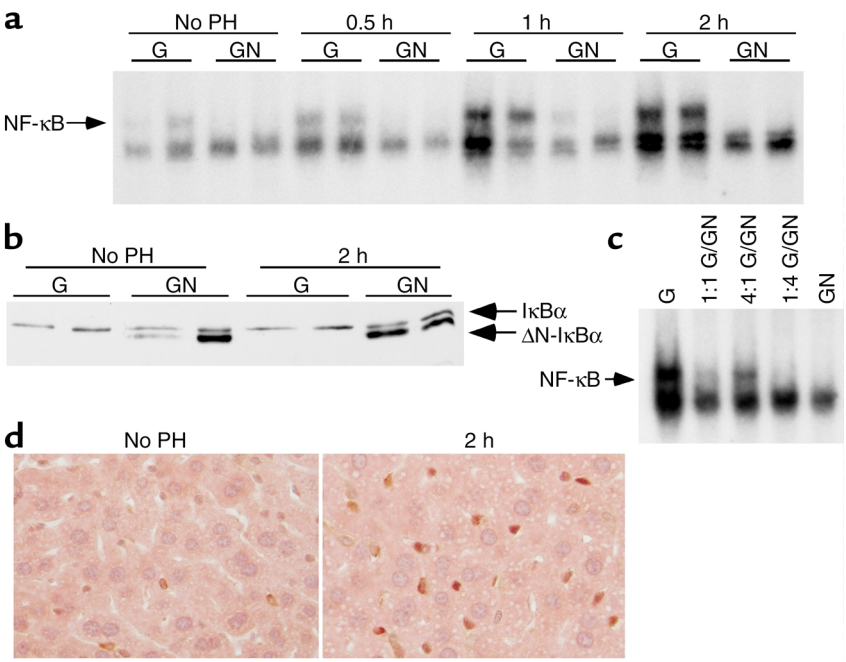

Figure 5
Analysis of NF-κB activation in mifepristone-treated mice after PH. (**a**) EMSA analysis of NF-κB DNA binding. Nuclear extracts were prepared from livers of mifepristone-treated single- or double-transgenic mice killed at the indicated times after PH. Five micrograms of nuclear protein was incubated with labeled NF-κB probe as described in Methods. Lanes represent individual animals. (**b**) IκBα Western blot of liver nuclear protein. Ten micrograms of liver nuclear extract protein used for EMSA shown in **a** was used for IκBα immunoblot. (**c**) Coincubation assay. Nuclear extract protein from 2-hour PH samples used in **a** were incubated alone or together at the indicated ratios with labeled NF-κB probe. (**d**) Histological analysis of p65 nuclear translocation in mifepristone-treated double-transgenic mice killed without PH or 2 hours after PH. Original magnification, ×400.

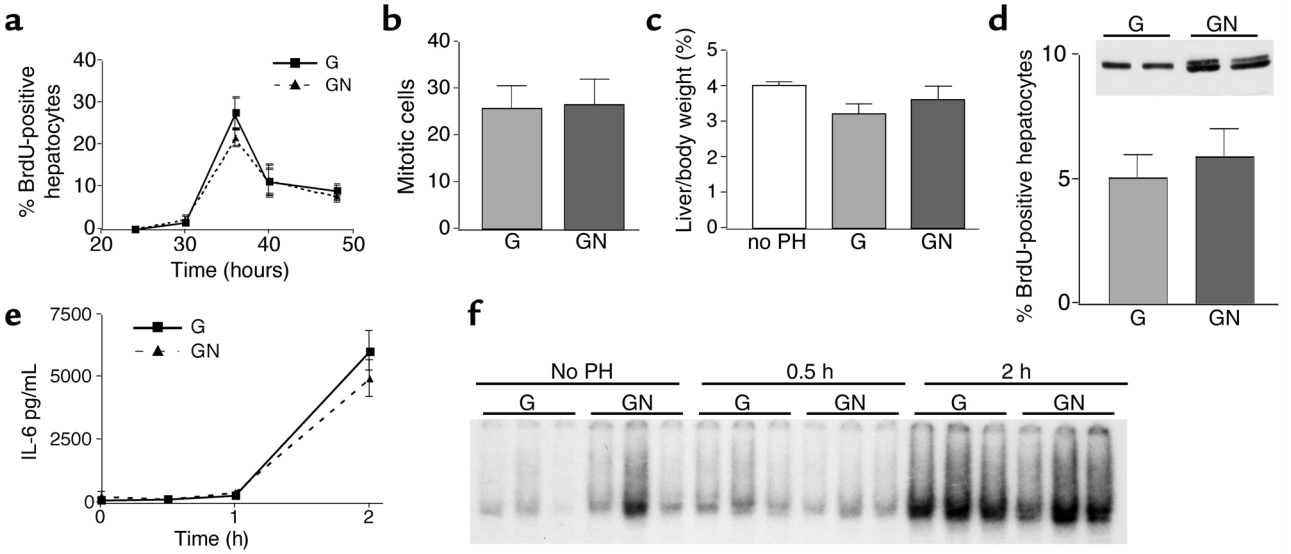

Figure 6

Expression of ΔN-IκBα in hepatocytes does not inhibit liver regeneration or IL-6 or STAT3 activation after PH. (**a**) Hepatocyte DNA synthesis. Mifepristone-treated single-transgenic and double-transgenic mice were injected with BrdU 2 hours prior to killing at the indicated times after PH. Liver sections were stained with an antibody to BrdU as described in Methods, and BrdU-positive hepatocyte nuclei were scored for 30 fields (×400) per slide. Four mice were analyzed per group, and data represent average percentage (± SEM) of BrdU-positive nuclei per group. (**b**) Mitotic index. Mitotic hepatocytes were counted in mifepristone-treated mice killed 48 hours after PH. Four mice were analyzed per group, and data represent average total number ± SEM of mitotic hepatocytes counted per 30 fields (×400). (**c**) Liver/body weight ratio. Liver and whole body weights were determined for untreated mice (no PH) or mifepristone-treated mice killed 2 weeks after PH. Three mice were used per group, and data represent average (± SEM) liver weight expressed as a percentage of whole body weight. (**d**) DNA synthesis at 48 hours after PH in mice injected with mifepristone at 12-hour intervals. (**e**) IL-6 ELISA. Serum was obtained from mifepristone-treated single- or double-transgenic mice killed at the indicated times after PH. Data represent average (± SEM) IL-6 levels per group. Three mice were analyzed per group. (**f**) STAT3 EMSA. Nuclear extracts were prepared from livers of mice described in **e**. Five micrograms of nuclear protein were analyzed for STAT3 DNA binding activity as described in Methods. Each lane represents an individual animal.

and 25 μg/kg TNF (Figure 7b). Additionally, a similar degree of IκBα degradation was observed with both doses (Figure 7c). The effect of TNF dose on activation of apoptosis was analyzed using both mifepristone-injected double-transgenic ΔN-IκBα mice and C57BL6 mice injected with D-galactosamine. In both systems, injection of 5 μg/kg TNF failed to activate hepatocyte apoptosis as measured by caspase-3 activity (Figure 7d) or histological evaluation (not shown). Instead, a fivefold higher dose of TNF was required to activate apoptosis in each of these experimental systems. We also analyzed release of liver enzymes, AST and ALT, in double-transgenic mice injected with mifepristone and TNF. Increased AST/ALT was observed only at the higher dose of TNF (Figure 7e). These data suggest that inhibition of NF-κB–dependent antiapoptotic genes alone is not sufficient to create an environment permissive for TNF-induced apoptosis in mouse hepatocytes. Instead, very high doses of TNF in combination with NF-κB inhibition are required to activate cell death.

Discussion

NF-κB is activated in the liver during the first several hours after PH (4, 5). While there are many studies on the role of NF-κB in liver regeneration, the separate contributions of hepatocyte and NPC NF-κB activation have not been examined. We developed a mouse system in which activation of NF-κB can be selectively and inducibly blocked in hepatocytes without affecting NPCs. Using this system, we show that blockage of hepatocyte NF-κB causes apoptosis after TNF injection but not after PH. We and others have previously shown that TNF causes apoptosis in cultured liver cells in which NF-κB activation is blocked (2, 3). Here, we demonstrate that hepatocytes in the intact mouse liver become sensitive to TNF-mediated apoptosis upon blockage of NF-κB translocation to the cell nucleus. However, NF-κB blockage is not an absolute determinant of TNF-induced apoptosis. On the contrary, while a dose of 5 μg/kg TNF activated NF-κB to a similar extent as 25 μg/kg TNF in control animals, apoptosis was induced only at the higher TNF dose in animals in which NF-κB activation was blocked. We conclude from these observations that NF-κB activation is not the only mechanism of hepatocyte protection against TNF-induced apoptosis. Our experiments suggest that NF-κB activation in hepatocytes of intact liver may be an important protection mechanism against high doses of TNF. However, in the regenerating liver after PH, it seems likely that levels of TNF do not reach the concentration threshold that may lead to apoptosis in hepatocytes in which NF-κB activation is blocked.

Additionally, the activation of NF-κB in NPCs and subsequent IL-6 production also contribute to antiapoptotic effects, as it has previously been shown that this cytokine has antiapoptotic as well as proliferative effects in the regenerating liver (32). Furthermore, upregulation of the delayed early gene *Bcl-x*$_L$ during liver regeneration (29) provides an NF-κB–independent antiapoptotic signal, since Bcl-x$_L$ mRNA increased greatly after PH but not after TNF injection. Given these observations, it is not unexpected that blockage of NF-κB activation in hepatocytes after PH without interference with activation in NPCs did not cause hepatocyte apoptosis.

Iimuro et al. reported that adenovirus-mediated expression of an IκBα super-repressor caused apoptosis after PH in rats (9). Expression of the mutant *IκBα* gene did not interfere with DNA replication after PH but caused massive apoptosis in cells in the G2 phase of the cell cycle. Mechanisms of protection from apoptosis might be different in rats and mice; however, the most likely explanation for the discrepancy between our results and those of Iimuro et al. is that we did not use an adenovirus vector to deliver the *IκBα* super-repressor gene. Instead, we used an inducible transgenic mouse system to express ΔN-IκBα specifically in hepatocytes. Adenovirus vectors can infect both parenchymal cells and NPCs of the liver (16, 33), and therefore delivery of the IκBα super-repressor through the viral vector probably prevented NF-κB activation in both hepatocytes and NPCs. Moreover, the adenoviral vectors themselves cause liver injury associated with the release of TNF and other cytokines as well as inducing DNA replication in nonhepatectomized animals as reported by Iimuro et al. (9) and Lieber et al. (16).

The combination of these effects complicates the analysis of the relationship between NF-κB activation and apoptosis in the regenerating liver. Recent experiments using the immunosuppressive fungal metabolite gliotoxin to inhibit NF-κB activation during liver regeneration in mice showed activation of apoptosis at 24 hours after PH and significant decreases in DNA replication at 48 hours (34). It will be important to determine whether gliotoxin blocks NF-κB activation in both parenchymal cells and NPCs and to exclude the possibility that this fungal metabolite has other effects that may block DNA replication independently of NF-κB. As shown by Lavon et al. (10) and the experiments reported here, it is clear that blockage of NF-κB activation in hepatocytes without interference with NPC NF-κB pathways causes hepatocyte apoptosis in vivo after administration of concanavalin A and high doses of TNF, respectively. On the other hand, in the regenerating liver, production of protective cytokines by NPCs as well as other mechanisms are sufficient to prevent these cells from undergoing apoptosis even if hepatocyte NF-κB activation is blocked.

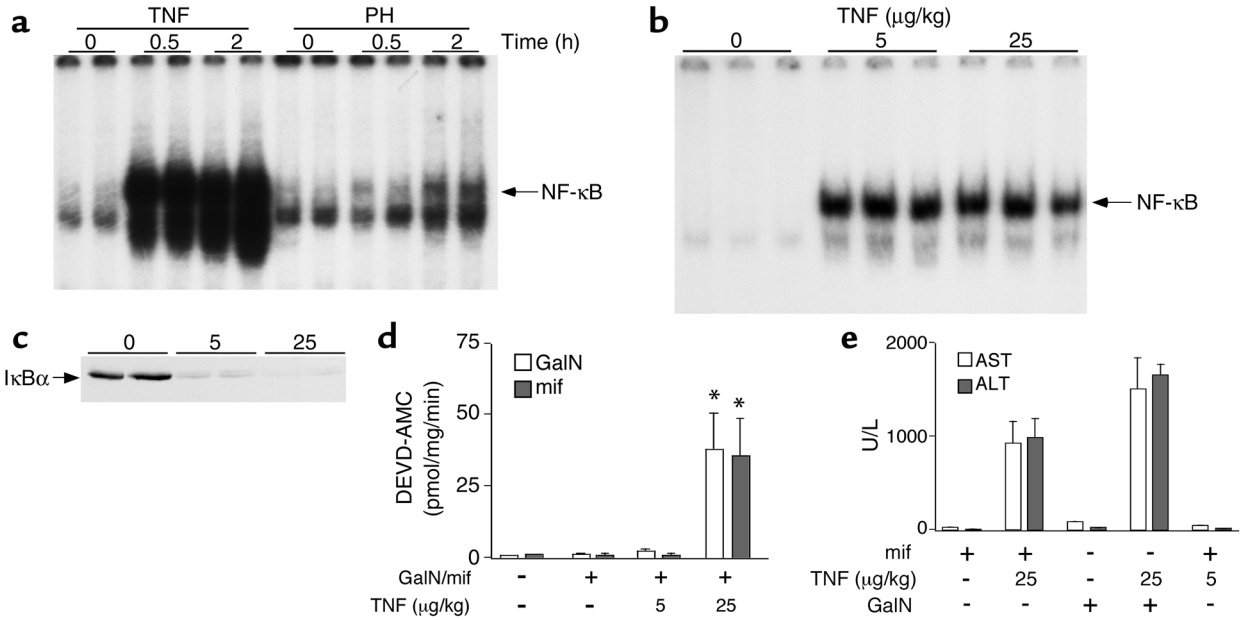

Figure 7

TNF-induced apoptosis occurs at a higher dose of TNF than that required to activate NF-κB. (**a**) Comparison by EMSA of NF-κB activation after TNF injection or PH. Nuclear protein was prepared from livers of mifepristone-treated single-transgenic mice killed at the indicated times after injection of 25 μg/kg TNF (left lanes) or PH (right lanes). (**b**) NF-κB EMSA. Nuclear extracts were prepared from livers of C57BL6 mice killed 30 minutes after injection with the indicated dose of TNF. (**c**) IκBα Western blot. Liver protein lysates were prepared from mice described in **b** and immunoblotted for IκBα. (**d**) Caspase-3 activity. Mice were killed 4 hours after treatment with the indicated dose of TNF in combination with either mifepristone (mif) or D-galactosamine (GalN). For mifepristone, double-transgenic mice were used. For D-galactosamine, C57BL6 mice were used. One hundred micrograms of liver protein lysates were incubated with fluorogenic DEVD-AMC as described in Methods. *$P < 0.05$ compared with vehicle controls. (**e**) AST/ALT activity. Serum was collected from mice killed 7 hours after treatment with TNF in combination with mifepristone or D-galactosamine, as described in **d**.

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