

Hepatocellular apoptosis is mediated by TNF α -dependent Fas/FasLigand cytotoxicity in a murine model of acute liver failure

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Abstract There is increasing evidence that the active contribution of hepatocytes to liver disease is strongly dependent on local cytokine environment. It has been shown in vitro that TNF α can enhance hepatocyte FasLigand (FasL)-mediated cytotoxicity. Here, we demonstrate that TNF α -induced apoptosis was associated with Fas and FasL upregulation and that a FasL-neutralizing antibody prevented TNF α -induced apoptosis. We further examined in vivo the relevance of the Fas/FasL pathway to hepatocellular apoptosis in a TNF α -driven model of acute liver failure. Livers of galactosamine/lipopolysaccharide (Gal/LPS)-exposed Fas wild-type mice highly expressed both Fas and FasL and revealed marked hepatocellular apoptosis that was almost completely blocked by soluble TNF α -receptor; this was also almost absent in Gal/LPS-exposed Fas lymphoproliferation mutant mice. Our data provide evidence for a direct link between TNF α and Fas/FasL in mediating hepatocyte apoptosis. Fratricidal death by Fas–FasL interactions of neighbouring hepatocytes may actively contribute to acute liver failure.

Keywords Caspase-3 · Flow cytometry · HepG2 cells · Liver enzymes · Microcirculation

Introduction

Acute liver failure (ALF) is a rare clinical syndrome associated with high mortality. Hepatic failure leads to a well-recognized pattern of clinical signs and symptoms, sometimes with rapid deterioration and progression to multi-organ failure [1]. Hepatic cell death is the leading cause of fatality in patients with ALF. The development of hepatic necrosis and apoptosis gives rise to severe hyperammonemia, hepatic encephalopathy, and life-threatening cerebral edema. In contrast with the well-documented morphological characterization of dying hepatocytes, the molecular pathways leading to the death of hepatocytes are not completely understood. A large amount of recent research indicates that a complex system, comprising death factors and death receptors, mediates cell death in liver disease. Our understanding of which cell death receptors are involved and how they function will become increasingly important for developing rational therapeutic strategies. In line with this, anti-apoptotic interventions have been stated to be among the most promising pharmacological options in the near future to ameliorate clinical liver disease [2].

Death receptors that have been assessed in purified liver cell preparations include Fas, Toll-like receptors (TLRs), and tumor necrosis factor receptors (TNFR)-1 and -2, as well as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors 1, 2, 3, and 4 [3]. Accordingly, the endogenous production of FasLigand (FasL) and TNF α has been implicated in mediating hepatic cell death in experimental models of hepatitis. Blockade of death receptor pathways has been shown to ameliorate liver disease to various degrees [4–8]. Within this context, however, the relative contribution of these pathways to hepatic injury is controversial and seems to depend

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significantly on the experimental models used. While in concanavalin A-induced hepatitis the contribution of TNF α to hepatic injury appears to predominate [5], other groups have failed to confirm the role of TNF α in this model and instead suggest a FasL-dependent pathway [9, 10]. In contrast with these findings, the galactosamine/lipopolysaccharide (Gal/LPS)-induced model of liver injury is reported to be mediated by neither Fas antigen [8] nor FasL [6]; however, it seems to unequivocally comprise the secretion of TNF α and its binding to TNFR-1, thereby mediating hepatic injury and lethality [11].

Most recently, hepatocytes have been recognized as cytotoxic effector cells under conditions of inflammation. Within this context, it was reported that hepatocytes exposed to TNF α exert cytotoxic activity via a FasL-mediated pathway and eliminate Fas-sensitive target cells [12]. Based on these findings, it is reasonable to hypothesize that under conditions of hepatic inflammation, such as Gal/LPS-induced ALF with TNF α release, hepatocyte Fas/FasL-dependent killing might contribute to liver injury in either a fratricidal or a suicidal manner [12, 13]. To address the relevance of upregulation of FasL and its cognate receptor in hepatocyte cytotoxic activity, we studied Fas/FasL expression and hepatocellular apoptosis in Gal/LPS-exposed Fas wild-type (Fas wt) and Fas lpr (lymphoproliferation) mutant (Fas lpr/lpr) mice and neutralized TNF α by application of a soluble TNF α -receptor (sTNF α -R).

Material and methods

Cytochemistry and flow cytometry of HepG2 cells

The human hepatoma cell line HepG2 was used for all in vitro experiments and was cultured as reported [12]. Cells were seeded in four-well Chamber Slides (LAB-TEK^T; Nunc, Wiesbaden, Germany) for cytochemical analysis and in six-well plates for flow cytometric analysis. After reaching confluence, cells were incubated with 500 U/mL human TNF α (hTNF α ; Sigma, Taufkirchen, Germany) or vehicle solution ($n = 4$ independent experiments each) for 6 h to induce apoptosis, in accordance with previously published work [12]. In an additional series of experiments, we simultaneously added 200 ng/mL mAb NOK-1 (BD Pharmingen, Heidelberg, Germany) to neutralize FasL.

For direct visualization of TNF α -induced apoptotic cell death cells were incubated with bisbenzimidazole (Hoechst 33342; Sigma) for an additional 15 min at room temperature (RT). The labeled cells were analyzed with a fluorescence microscope (Axioskop 40; Zeiss, Jena, Germany).

Flow cytometric analysis of cell apoptosis was performed by means of 7-AAD staining as described by Philpott et al. [14]. After cell detachment by trypsinization

and washing, cells were fixed in 70% methanol at -20°C for 20 min, followed by incubation with 25 $\mu\text{g}/\text{ml}$ RNase A (Roth, Karlsruhe, Germany) for 15 min at 37°C and staining with 7-AAD (200 $\mu\text{g}/\text{ml}$; Molecular Probes, Eugene, OR, USA) for 30 min at RT. Apoptotic cells were assessed by quantitative analysis of the sub G_0/G_1 peak using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

For evaluation of concomitant Fas and FasL expression, fixed cells were incubated with rabbit polyclonal anti-Fas (1:100, M-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal anti-FasL (1:100, C-20; Santa Cruz Biotechnology), or with an isotype-specific immunoglobulin (1:200; Santa Cruz Biotechnology) as a negative control antibody for 30 min at 37°C . After two washing steps, goat anti-rabbit-FITC antibody (1:200; Santa Cruz Biotechnology) was applied for 30 min at 37°C . The labeled cells were analyzed by means of FACScan flow cytometry.

Model of ALF and experimental groups

Male Fas wild-type mice (C57BL/6J; Fas wt) (Charles River Laboratories, Sulzfeld, Germany) and Fas lpr mutants (B6-lpr/lpr(Fas); Fas lpr/lpr) (Jackson Laboratory, Maine, USA) were used at 8–10 week of age with a body weight of approximately 20 g. Animals were provided with water and standard laboratory chow ad libitum. The experimental protocol was approved by the local committee and all animals received humane care according to the German legislation on protection of animals and the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23 revised 1985).

For induction of ALF, mice were injected with Gal (720 mg/kg body weight intraperitoneally [bw ip]; Sigma) and LPS (10 $\mu\text{g}/\text{kg}$ bw ip, serotype 0128:B12; Sigma) and were studied 6 h thereafter. Concentrations of Gal and LPS were used in accordance with work published previously by our [15, 16] and other groups [8, 17]. To address the contribution of TNF α in Gal/LPS-induced ALF, animals were pretreated with sTNF α -R (recombinant human TNFR Fc fusion protein, etanercept, 100 $\mu\text{g}/\text{kg}$ bw ip, Enbrel; Wyeth Pharma GmbH, Münster, Germany) or equivalent volumes of vehicle solution (0.9% saline 1 mL/kg bw ip) 1 h prior to ALF induction. Although it is of human origin, etanercept has been characterized as a TNF α -blocking drug that also prevents rheumatoid arthritis in mice [18].

Intravital fluorescence microscopy

For in vivo analysis of hepatocellular apoptosis, intrahepatic leukocyte accumulation, and sinusoidal perfusion failure, fluorescence microscopy was performed 6 h after

Gal/LPS exposure in ketamine/xylazine-anesthetized animals (75/25 mg/kg bw ip) in accordance with work previously published by our group [15, 16, 19].

Sampling and assays

After in vivo microscopy, animals were exsanguinated by puncture of the vena cava inferior for immediate separation of EDTA plasma. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured spectrophotometrically as indicators for hepatocellular disintegration and necrosis. Liver tissue was sampled for subsequent RT-PCR, Western blot analysis, histology, and immunohistochemistry.

RT-PCR analysis of liver tissue

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. cDNA synthesis was performed using Superscript II RT (Invitrogen, Karlsruhe, Germany) in accordance with the manufacturer's instructions. PCR for murine Fas, FasL, and GAPDH was performed in a 20 μ L reaction volume containing 1 \times reaction puffer, 20 μ M dNTPs, and 5,000 U/mL *Taq* polymerase. Samples were incubated with respective primers (Table 1) for either 30 (FasL and GAPDH) or 35 (Fas) cycles (denaturation for 30 s at 94°C; annealing for 30 s at 58°C (Fas), 56°C (FasL), or 54°C (GAPDH); and elongation for 30 s at 72°C) using a thermocycler (Eppendorf Mastercycler Gradient, New York, NY, USA). PCR products were analyzed on a 1% agarose gel (peq-GOLD Universal Agarose; peQLab Biotechnologie, Erlangen, Germany) containing 0.5 μ g/ml ethidium bromide. Signals were densitometrically assessed (Quantity One, ChemiDoc XRS System; Bio-Rad Laboratories, Munich, Germany) and normalized to the GAPDH signals.

Western blot analysis of liver tissue

For Western blot analysis of protein levels of cleaved caspase-3, Fas, and FasL, liver tissue was homogenized in lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 0.5% Triton-X 100, 0.02% NaN₃, 0.2 mM PMSF, and protease inhibitor cocktail), incubated for 30 min on ice and

centrifuged for 15 min at 10,000g. Protein content were assayed by the BCA method (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard. Per lane, either 40 μ g of protein (cleaved caspase-3 and TLR-4) or 20 μ g of protein (Fas and FasL) was separated on a 12% SDS gel and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). After blocking with 2% BSA (Sigma), membranes were incubated for 2 h at RT with a rabbit polyclonal anti-cleaved caspase 3 (1:1,000, Asp 175; Stressgen Biotech, San Diego, CA, USA), rabbit polyclonal anti-FasL (1:500, C-20; Santa Cruz Biotechnology), rabbit polyclonal anti-Fas (1:500, M-20; Santa Cruz Biotechnology), and goat polyclonal anti-TLR-4 (1:1000, M-16; Santa Cruz Biotechnology) followed by a secondary peroxidase-linked goat anti-rabbit antibody (cleaved caspase-3 and FasL: 1:2,000 and Fas: 1:5,000; Cell Signaling Technology, Boston, MA, USA) and donkey anti-goat antibody (TLR-4: 1:7,500; Santa Cruz Biotechnology). Protein expression was visualized by means of luminol-enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech, Freiburg, Germany) and digitalized with the ChemiDoc XRS System (Bio-Rad Laboratories GmbH). Signals were densitometrically assessed (Quantity One; Bio-Rad Laboratories) and normalized to β -actin signals (mouse monoclonal anti- β -actin antibody: 1:20,000; Sigma).

Histology and immunohistochemistry of liver tissue

For hematoxylin and eosin (H&E) staining for the immunohistochemical study of cleaved caspase-3 and for analysis of TUNEL-positive hepatocytes, we used protocols in accordance with work previously published by our group [15, 16]. For immunohistochemical staining of Fas and FasL expression serial sections of liver tissue were incubated with primary antibodies overnight at 4°C (polyclonal anti-Fas (1:50, M-20) and polyclonal anti-FasL (1:50, C-20); both Santa Cruz Biotechnology). Universal LSAB[®] kits (System-HRP and System-AP; DakoCytomation, Dako, Hamburg, Germany) were used according to the manufacturer's instructions for the development of Fas with DAB chromogen and of FasL with fuchsin chromogen. The sections were counterstained with hemalaun and analyzed with a light microscope (Olympus BX51, Hamburg, Germany).

Table 1 List of primers used for RT-PCR

Transcript	Forward primer (5'–3')	Reverse primer (5'–3')
Fas	TAT CAA GGA GGC CCA TTT TG	GGT CAG GGT GCA GTT TGT TT
FasL	TGG TTG GAA TGG GAT TAG GA	TTA AAT GGG CCA CAC TCC TC
GAPDH	AAC GAC CCC TTC ATT GAC	TTC ACG ACA TAC TCA GCA C

Statistical analysis

All data are expressed as mean \pm SEM. Statistical differences among groups were determined using an unpaired Student's *t*-test. Data were considered significant when $P < 0.05$. Statistical analysis was performed using the Sigma Stat and Sigma Plot software package (Jandel Scientific, San Rafael, CA, USA).

Results

Cytochemistry and flow cytometry of HepG2 cells

Under basal conditions, flow cytometry of HepG2 cultures demonstrated that 4% of the cells underwent apoptosis (Fig. 1a, b) with a negligible fraction concomitantly expressing Fas ($\sim 1\%$) or FasL ($\sim 5\%$). In contrast, TNF α exposure of HepG2 cells resulted in a huge rise in apoptotic cells ($57 \pm 11\%$), of which $11 \pm 2\%$ expressed Fas, but $44 \pm 8\%$ expressed FasL (Fig. 1c). Noteworthy, adding the FasL-neutralizing antibody NOK-1 reduced TNF α -induced apoptosis to $9 \pm 1\%$ (Fig. 1b), of these apoptotic cells, only $20 \pm 3\%$ now expressed FasL (Fig. 1c). These findings underscore the involvement of the Fas/FasL pathway in the TNF α -dependent apoptosis of HepG2 cells.

Expression of Fas and FasL in Gal/LPS-exposed livers

RNA analysis of tissue from Gal/LPS-exposed livers showed a 1.4-fold increase in Fas expression and a 2-fold increase in FasL expression in Fas wt mice when compared with controls, i.e., animals without Gal/LPS challenge (Fig. 2a, b). TNF α blockade elicits a reduction in Fas and FasL mRNA expression of $\sim 30\%$ in Fas wt mice (Fig. 2a, b). In Fas lpr/lpr mice Fas mRNA was not detectable (Fig. 2a), but FasL mRNA expression was also upregulated after Gal/LPS exposition (Fig. 2b).

Fig. 1 Representative UV fluorescence images (a, upper) of bis-benzimide-stained HepG2 cells as well as images of the corresponding fields in trans-illumination technique (a, lower) upon hTNF α exposure (original magnification 500 \times). Note the typical signs of apoptotic HepG2 cells, i.e., nuclear fragmentation and condensation. Flow cytometric analysis of HepG2 cells (b) upon induction of apoptosis by human TNF α exposure and additional treatment with the FasL-neutralizing mAb NOK-1. Percentage of apoptotic HepG2 cells expressing either Fas or FasL (c). Values are given as mean \pm SEM; $n = 4$ independent experiments; unpaired Student's *t*-test, including Bonferroni correction to compensate for multiple comparisons; * $P < 0.05$ vs. unstimulated, # $P < 0.05$ vs. hTNF α

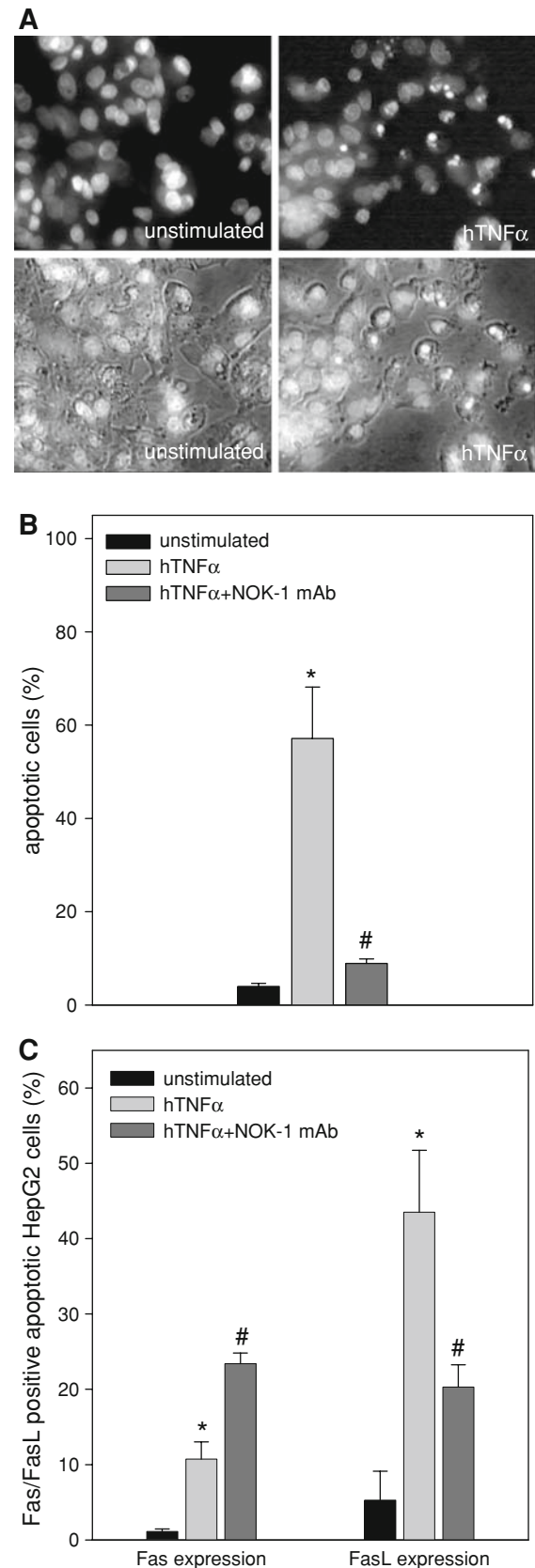


Fig. 2 Representative RT-PCR gels and densitometric analysis of Fas (a) and FasL (b) mRNA levels in livers of Fas wt ($n = 8$) and Fas lpr/lpr ($n = 8$) mice. Signals were corrected using GAPDH signal. Animals were injected with Gal and LPS for induction of ALF (–6 h) and pretreated (–1 h) with either soluble recombinant TNF α -R (sTNF α -R; $n = 4$) or equivalent volumes of vehicle solution (vehicle; $n = 4$). Control represents animals without induction of ALF receiving only isotonic saline. Values are given as mean \pm SEM; unpaired Student's t -test; # $P < 0.05$ vs. vehicle; n.d. = not detectable

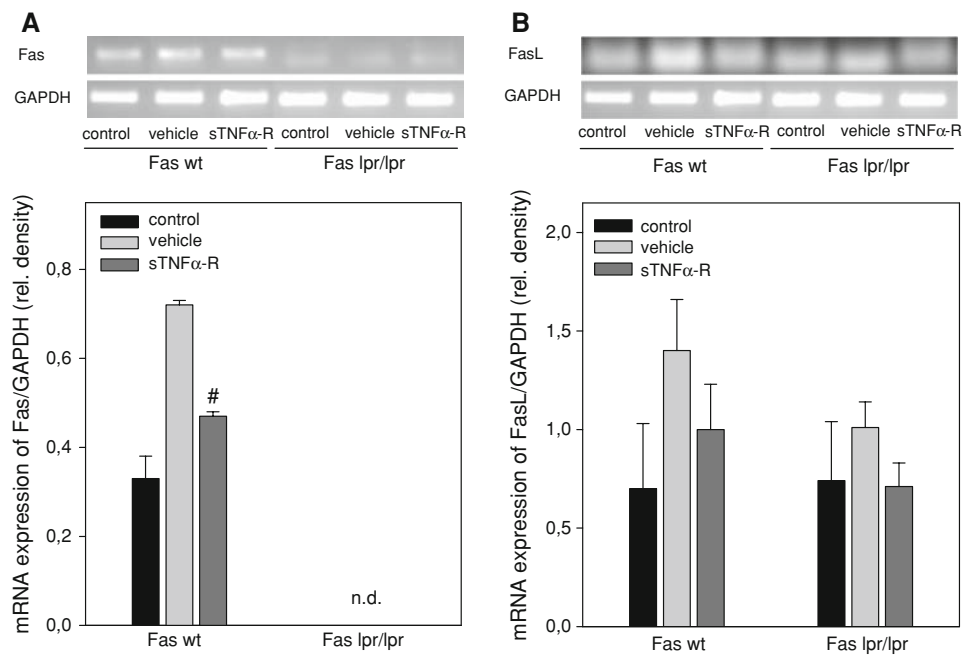
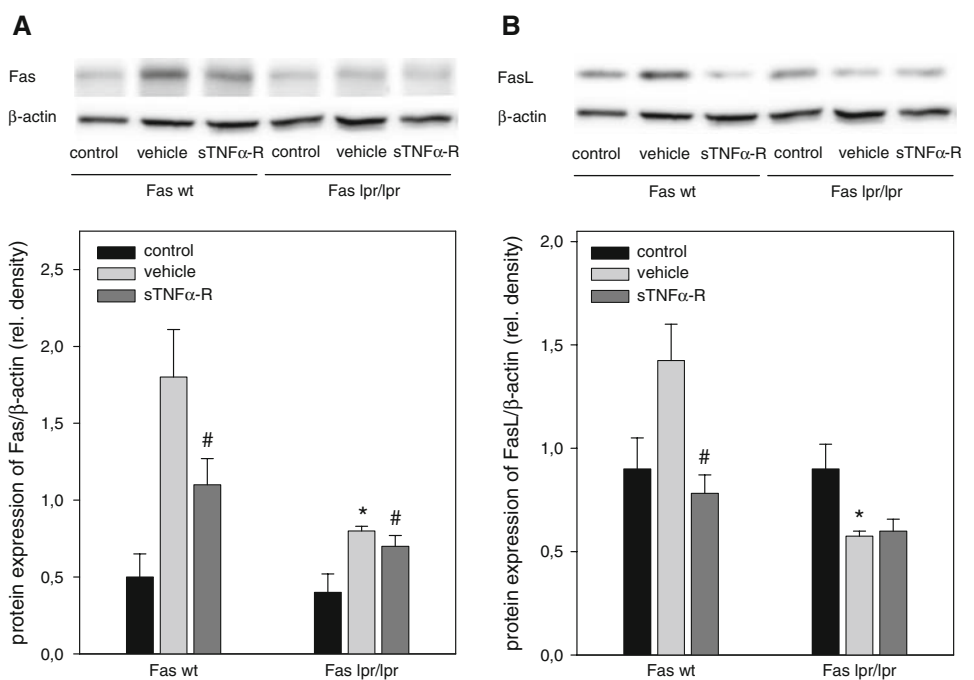


Fig. 3 Representative Western blots and densitometric analysis of Fas (a) and FasL (b) protein levels in livers of Fas wt ($n = 8$) and Fas lpr/lpr animals ($n = 8$). Signals were corrected using β -actin signal. Animals were injected with Gal and LPS for induction of ALF (–6 h) and pretreated (–1 h) with either soluble recombinant TNF α -R (sTNF α -R; $n = 4$) or equivalent volumes of vehicle solution (vehicle; $n = 4$). Control represents animals without induction of ALF receiving only isotonic saline. Values are given as mean \pm SEM; unpaired Student's t -test; * $P < 0.05$ vs. Fas wt; # $P < 0.05$ vs. vehicle



Protein analysis of Gal/LPS-exposed liver tissue showed a 3.6-fold increase in Fas expression and a 1.6-fold increase in FasL expression in Fas wt mice compared with controls, and revealed a marked reduction in both Fas (–39%) and FasL (–50%) upon TNF α blockade in Fas wt mice (Fig. 3a, b), indicating the dependence of the Fas/FasL pathway on TNF α during ALF. Fas lpr/lpr mice exhibited significantly lower levels of Fas and FasL compared with those found in Fas wt mice, and showed a significant reduction in Fas, but not in FasL, upon TNF α neutralization (Fig. 3a, b).

Additionally, immunohistochemical staining of Fas and FasL of serial liver tissue sections revealed a marked hepatocellular expression of the receptor and its ligand in the Gal/LPS-exposed Fas wt mice (Fig. 4, left panels), while only a weak staining of both Fas and FasL was detectable in the respective Fas lpr/lpr mice (Fig. 4, right panels). Noteworthy, fratricidal cell death by Fas–FasL interaction is underscored by the fact that Fas positive hepatocytes were found in the direct neighbourhood of hepatocytes which were FasL positive.

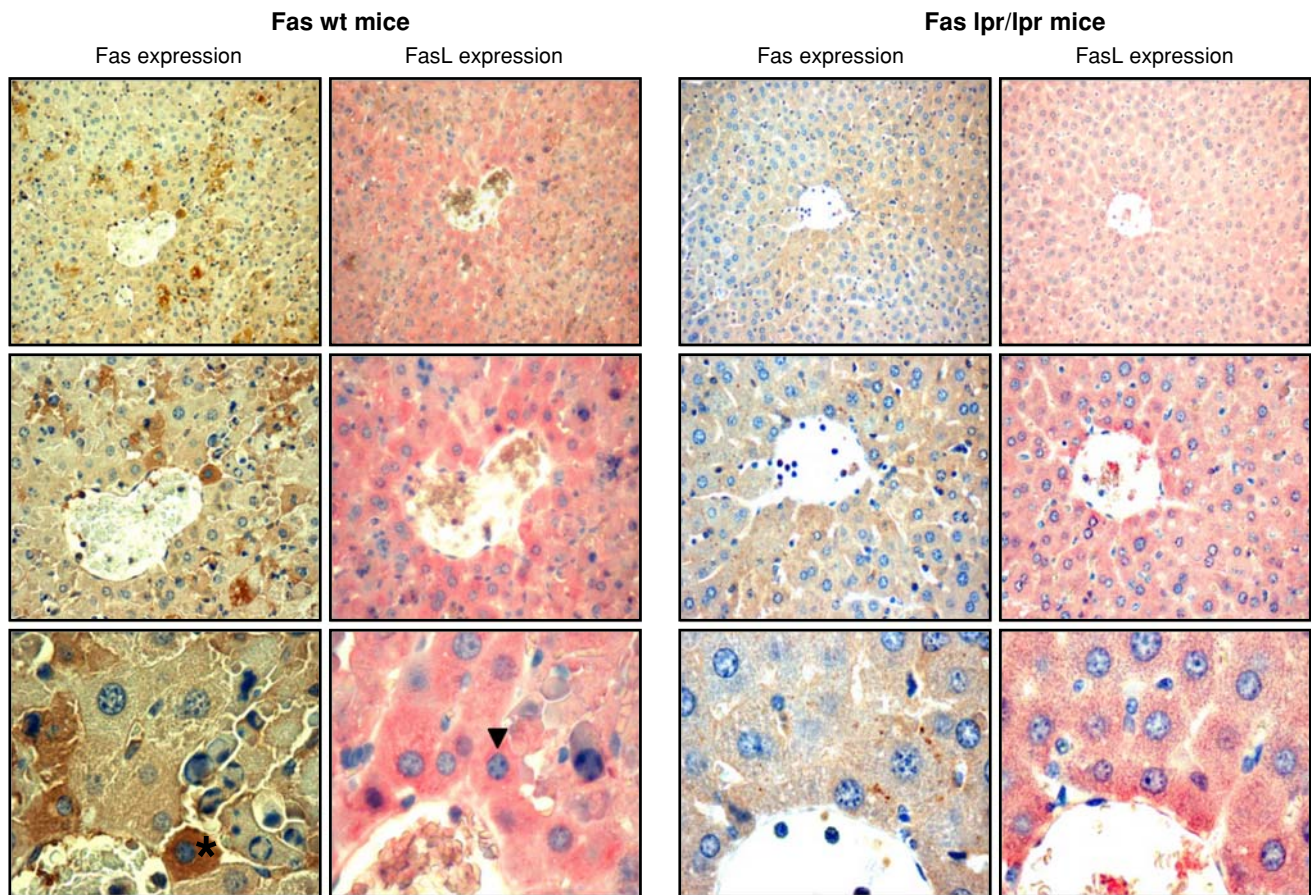


Fig. 4 Representative light microscopic images (upper panels, original magnification 200 \times ; middle, 400 \times ; lower, 1,000 \times) of immunohistochemical staining of Fas (DAB brown staining) and FasL (fuchsin red staining) in serial liver tissue sections of Fas wt (left panels) and Fas lpr/lpr mice (right panels). Note the marked hepatocellular expression of either Fas (asterisk) or FasL (arrow

head) of neighbouring hepatocytes in Gal/LPS-exposed Fas wt mice (left panel, 1,000 \times) and the nuclear chromatin fragmentation of the FasL positive hepatocyte as a sign of apoptosis indicating fratricidal cell death by Fas–FasL interaction. In contrast, there is only weak staining of both Fas and FasL in the respective Fas lpr/lpr mice (right panel)

Furthermore, Gal/LPS exposure with and without sTNF α -R revealed a 2-fold rise in TLR-4 protein expression in the liver tissue of Fas wt mice compared with the control (Fig. 5). In contrast, Gal/LPS-exposed Fas lpr/lpr mice exhibited only \sim 10% of the TLR-4 expression seen in Fas wt mice (Fig. 5).

Fas lpr/lpr mice showed a decrease in ALF-associated apoptotic cell death

An analysis of apoptotic cell death after Gal/LPS exposure is provided in Figs. 6 and 7, including in vivo microscopy (Fig. 6) and TUNEL histochemistry (Fig. 7) of hepatocellular apoptosis.

Fas wt mice exposed to Gal/LPS were characterized by a high number of apoptotic hepatocytes, while Fas lpr/lpr mice were markedly protected against ALF-associated apoptotic liver injury (Fig. 6). Confirming this, TUNEL histochemistry of liver tissue from Fas lpr/lpr mice with

ALF showed a more than 60% reduction in TUNEL-positive cells (Fig. 7). Gal/LPS-induced liver injury was found to be responsive to TNF α blockade, because pre-treatment with sTNF α -R resulted in nearly complete inhibition of apoptosis in Fas wt mice with a negligible low number of either bisbenzimid-stained hepatocytes, exhibiting nuclear chromatin condensation and fragmentation (Fig. 6), or TUNEL-positive hepatocytes (Fig. 7). Of utmost interest, the extent of hepatocellular apoptosis in Gal/LPS-challenged and vehicle-treated Fas lpr/lpr mice was significantly higher than that in Gal/LPS-challenged and TNF α -blocked Fas wt animals. This indicates that both the TNF α and the Fas/FasL pathway contribute to apoptotic injury in ALF. In further support of this view, TNF α neutralization in Fas lpr/lpr mice reduced the number of apoptotic hepatocytes to values similar to those found in TNF α -blocked Fas wt mice (Figs. 6, 7).

In support of these data, immunohistochemical and protein analysis of cleaved caspase-3 (Fig. 8) revealed less

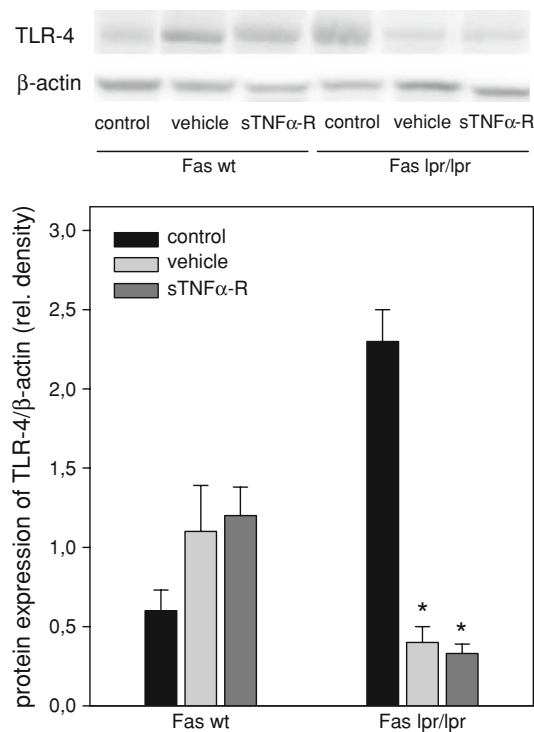


Fig. 5 Representative Western blots and densitometric analysis of TLR-4 protein levels in livers of Fas wt ($n = 8$) and Fas lpr/lpr animals ($n = 8$). Signals were corrected using β -actin signal. Animals were injected with Gal and LPS for induction of ALF (-6 h) and pretreated (-1 h) with either soluble recombinant TNF α -R (sTNF α -R; $n = 4$) or equivalent volumes of vehicle solution (vehicle; $n = 4$). Control represents animals without induction of ALF receiving only isotonic saline. Values are given as mean \pm SEM; unpaired Student's t -test; * $P < 0.05$ vs. Fas wt

activation of the effector caspase in Fas lpr/lpr vs. Fas wt mice after Gal/LPS challenge and treatment with the vehicle (control). Additionally, Western immunoblots for cleaved caspase-3 confirmed the dependency of Gal/LPS-induced ALF on TNF α , as shown by the marked decrease in cleaved caspase-3 protein upon pretreatment with sTNF α -R in Fas lpr/lpr, but most particularly in Fas wt mice (Fig. 8). Furthermore, the blockade of TNF α in Fas wt mice was more effective with respect to preventing apoptosis than was the Fas lpr mutation, as indicated by the significantly higher cleavage of caspase-3 protein in these animals after Gal/LPS exposure or treatment with the vehicle (Fig. 8).

Fas lpr mutation and TNF α blockade ameliorated hepatic microcirculatory dysfunction and liver tissue disintegration

Hepatic microcirculation analysis by fluorescence microscopy is given in Fig. 9. In the Gal/LPS-treated Fas wt mice, in vivo microscopy of the livers revealed characteristic features of acute injury, including increased sinusoidal

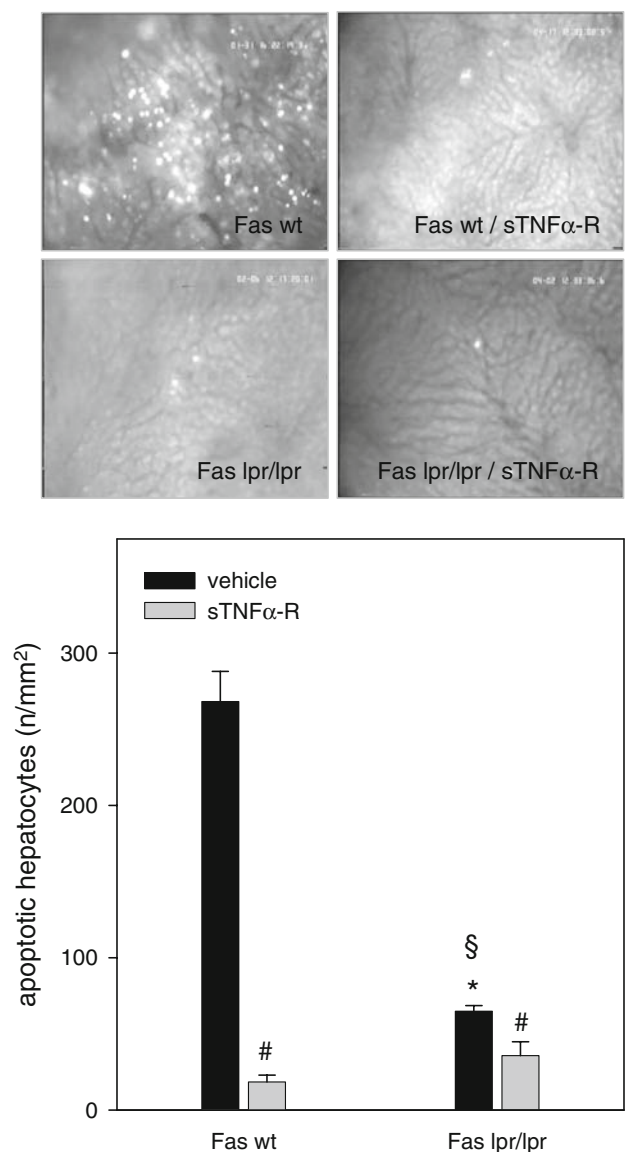


Fig. 6 Representative fluorescent microscopic images (upper panel, original magnification 200 \times) and quantitative analysis (lower panel) of apoptotic hepatocytes in Fas wt ($n = 14$) and Fas lpr/lpr animals ($n = 14$). Animals were injected with Gal and LPS for induction of ALF (-6 h) and pretreated (-1 h) with either soluble recombinant TNF α -R (sTNF α -R; $n = 7$) or equivalent volumes of vehicle solution (vehicle; $n = 7$). Hepatocellular apoptosis was assessed by in vivo fluorescence microscopy and bisbenzimidazole staining. Compare the high numbers of individual hepatocytes with condensation, but also fragmentation and/or margination of nuclear chromatin, characteristic signs of apoptotic cell death in Fas wt mice (upper left image), with the few apoptotic hepatocytes found in Fas wt mice upon TNF α blockade (upper right image), and in Fas lpr/lpr mice (lower left and right images). Values are given as mean \pm SEM; unpaired Student's t -test; * $P < 0.05$ vs. Fas wt; # $P < 0.05$ vs. vehicle; § $P < 0.05$ vs. Fas wt/sTNF α -R

leukocyte accumulation and pronounced perfusion failure of up to 59% nonperfused sinusoids (Fig. 9a, b). Application of sTNF α -R in Fas wt mice and Fas lpr mutation per se

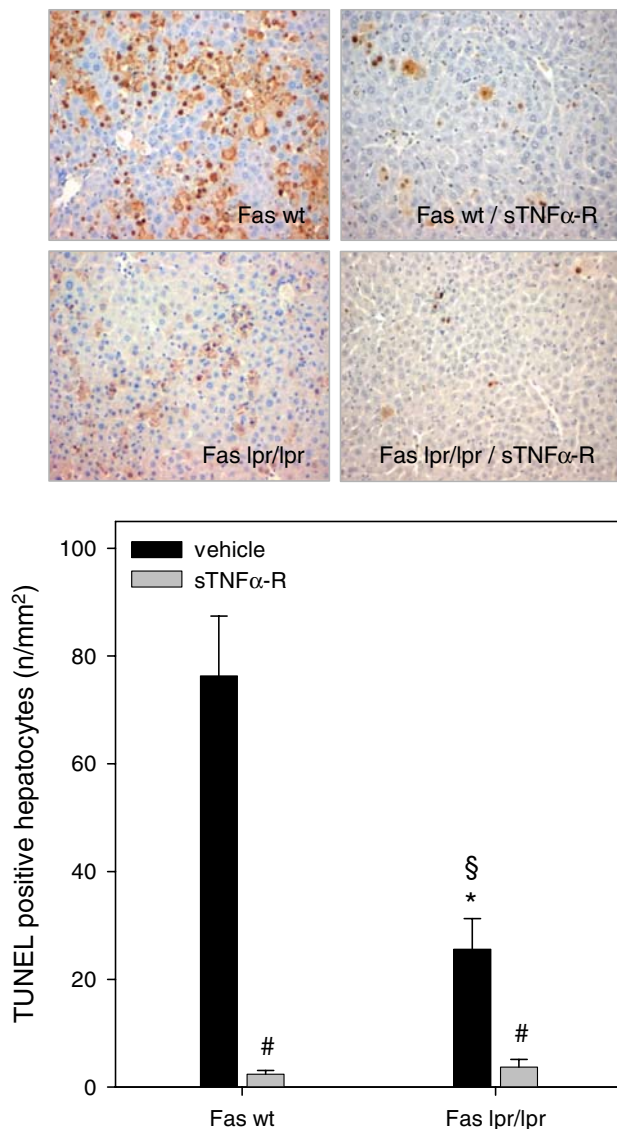


Fig. 7 Representative light microscopic images (*upper panel*, original magnification 200 \times) and quantitative analysis (*lower panel*) of TUNEL histochemistry for apoptotic hepatocytes in Fas wt ($n = 14$) and Fas lpr/lpr animals ($n = 14$). Animals were injected with Gal and LPS for induction of ALF (-6 h) and pretreated (-1 h) with either soluble recombinant TNF α -R (sTNF α -R; $n = 7$) or equivalent volumes of vehicle solution (vehicle; $n = 7$). Note the high numbers of TUNEL-positive hepatocytes in Fas wt mice (upper left image) and the marked reduction in cell apoptosis in Fas wt mice upon TNF α blockade (upper right image), and in Fas lpr/lpr mice (lower left and right images). Values are given as mean \pm SEM; unpaired Student's *t*-test; * $P < 0.05$ vs. Fas wt; # $P < 0.05$ vs. vehicle; § $P < 0.05$ vs. Fas wt/sTNF α -R

was associated with a reduction in inflammatory leukocyte stasis and microvascular perfusion failure (Fig. 9a, b).

H&E staining of liver tissue specimens from Gal/LPS-exposed Fas wt mice exhibited disruption of the general architecture and microvascular disintegration, as well as tissue apoptosis and necrosis (Fig. 10a). In line with the

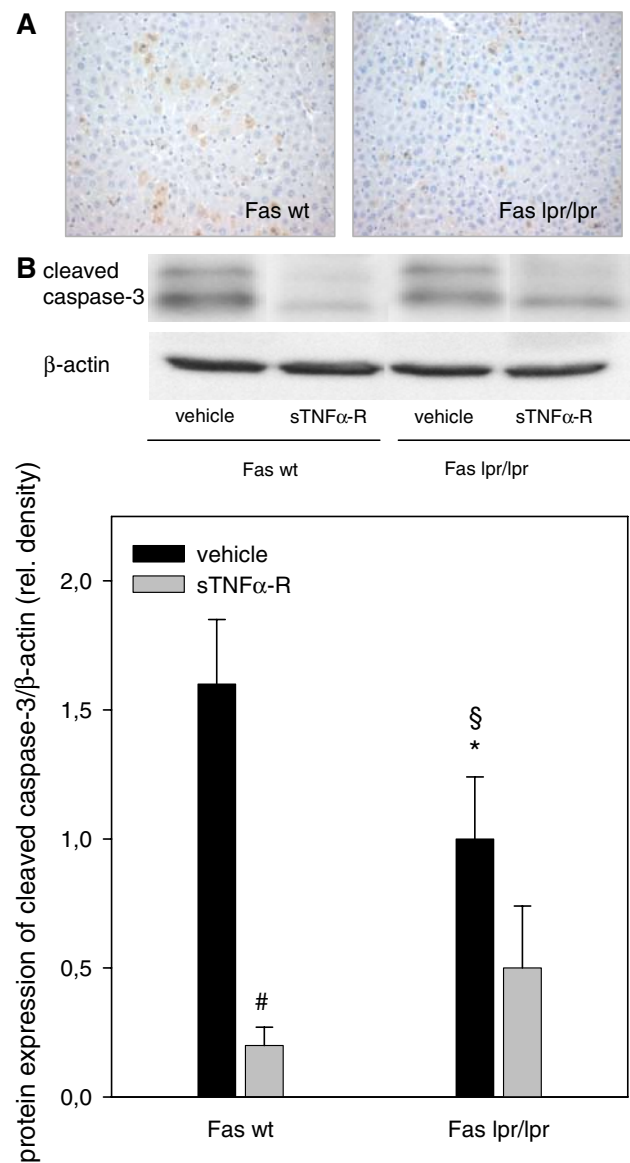
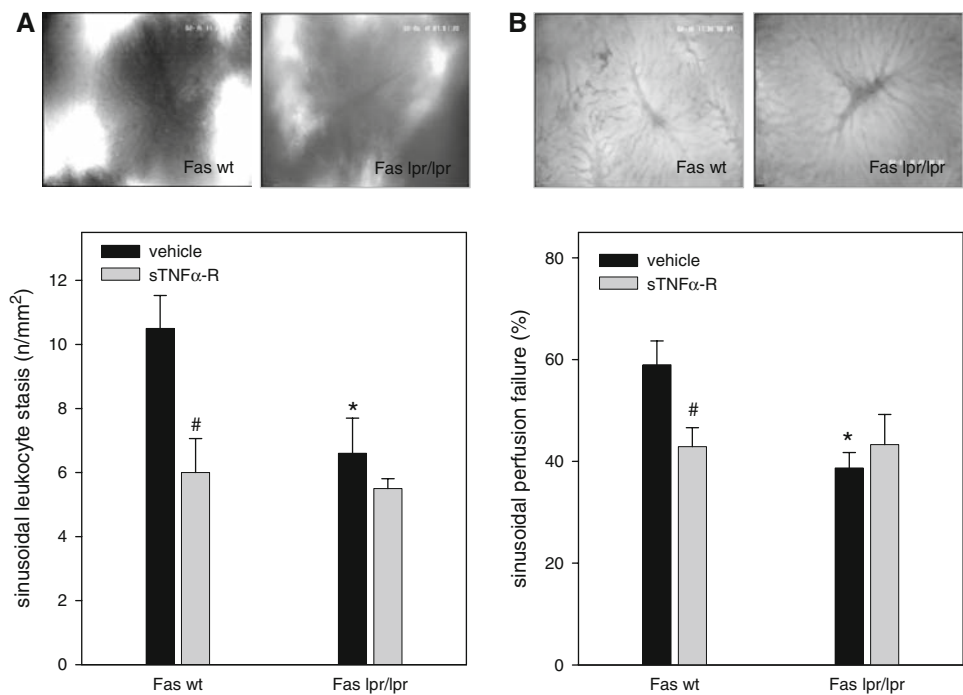


Fig. 8 Representative light microscopic images (*a, upper panel*, original magnification 200 \times) and Western blot, as well as densitometric analysis (*b*), of cleaved caspase-3 protein levels in livers of Fas wt ($n = 8$) and Fas lpr/lpr animals ($n = 8$). Animals were injected with Gal and LPS for induction of ALF (-6 h) and pretreated with either soluble recombinant TNF α -R (sTNF α -R; $n = 4$) or equivalent volumes of vehicle solution (vehicle; $n = 4$). Values are given as mean \pm SEM; unpaired Student's *t*-test; * $P < 0.05$ vs. Fas wt; # $P < 0.05$ vs. vehicle; § $P < 0.05$ vs. Fas wt/sTNF α -R

histopathology results, liver injury was reflected by high transaminase activities in plasma (Fig. 10b, c). Liver sections obtained from Fas wt mice with TNF α blockade and Fas lpr/lpr mice per se were different in appearance, often exhibiting grossly retained general architecture and lacking evidence of major morphological injury (Fig. 10a). Accordingly, transaminase levels were significantly reduced in these animals (Fig. 10b, c), although there were still significantly higher AST and ALT values in vehicle- and sTNF α -R-treated Fas lpr/lpr

Fig. 9 Representative fluorescent microscopic images (original magnification 200 \times) and quantitative analysis of hepatic sinusoidal leukocyte stasis (a) and sinusoidal perfusion failure (b) in Fas wt ($n = 14$) and Fas lpr/lpr animals ($n = 14$). Animals were injected with Gal and LPS for induction of ALF (-6 h) and pretreated (-1 h) with either soluble recombinant TNF α -R (sTNF α -R; $n = 7$) or equivalent volumes of vehicle solution (vehicle; $n = 7$). Values are given as mean \pm SEM; unpaired Student's t -test; * $P < 0.05$ vs. Fas wt; # $P < 0.05$ vs. vehicle



lpr animals when compared with transaminase levels in the TNF α -blocked Fas wt mice (Fig. 10).

Discussion

In this study, we showed that the upregulation of Fas and FasL in Gal-sensitized mice upon exposure to LPS is dependent upon TNF α ; this was shown by application of sTNF α -R, which neutralized TNF α , partially preventing inflammatory Fas and FasL expression. We further demonstrated the relative contribution of TNF α and the Fas/FasL pathway to hepatic injury and observed that both Fas lpr mutation and blockade of endogenous TNF α limited hepatic injury, although we found that neutralization of TNF α was more potent in preventing Gal/LPS-induced hepatocellular apoptosis. This led us to conclude that hepatocyte apoptosis is indeed driven mainly by TNF α ; however, it necessarily includes Fas/FasL cytotoxicity in the present murine model of ALF. In addition, TNF α -induced apoptosis of HepG2 cells was substantially reduced by a neutralizing anti-FasL antibody. Thus, we propose that TNF α -dependent hepatocyte apoptosis involves activation of Fas by upregulation of FasL, and that Fas/FasL-mediated cytotoxicity of hepatocytes is of considerable relevance in the present TNF α -driven model of liver injury.

Methodological considerations

As reported by our group [15, 16] and other groups [8, 17], we used the administration of LPS to Gal-sensitized mice

as an experimental model for endotoxic or septic shock [8, 11], although other models for liver injury-like application of TNF α to Gal-sensitized mice would also have been appropriate [17]. In contrast to previous predominantly T-cell-mediated models of hepatitis, Gal/LPS-induced liver injury is presumed to be principally a macrophage/monocyte-mediated model [20], and presents, in our hands, with high apoptotic cell death, deterioration of microcirculation, and transaminase release. We focused in the current work on mediation of hepatocellular apoptosis, which was confirmed by several independent methods, such as in vivo fluorescence microscopy and TUNEL histochemistry, as well as protein analysis and immunohistochemistry of caspase-3 cleavage.

While other groups have used mice with a spontaneous mutation in FasL or applied soluble Fas fusion protein [6], we used mice with the lpr mutation in the Fas gene to address the contribution of the Fas/FasL pathway in Gal/LPS-induced liver injury. In these mice, insertion of an early transposable element (ETn) in intron 2 of Fas antigen results in a loss-of-function mutation [21] with the consequence of a small but distinct rearrangement of the Fas antigen gene [22]. Although we were not able to detect Fas mRNA transcription, it has been reported that a small amount of transcript can be read through the ETn region and spliced correctly [23]. This might explain the detection of Fas protein in the Fas lpr/lpr mice of the present study, similar to what has been described by others [24].

Although we were aware of the possibility that liver tissue could be contaminated by infiltrating cells bearing Fas and FasL, we analyzed Fas and FasL by RT-PCR and

Fig. 10 Representative H&E-stained images (**a**, original magnification 400 \times) and plasma AST (**b**) and ALT activities (**c**) in Fas wt ($n = 14$) and Fas lpr/lpr animals ($n = 14$). Animals were injected with Gal and LPS for induction of ALF (-6 h) and pretreated (-1 h) with either soluble recombinant TNF α -R (sTNF α -R; $n = 7$) or equivalent volumes of vehicle solution (vehicle; $n = 7$). **a** Note the deterioration of hepatic morphology, including tissue apoptosis (arrow-heads) and necrosis (arrows) in Gal/LPS-exposed Fas wt mice (left upper image), while tissue integrity was almost entirely maintained in liver specimens from animals in the other groups. Values are given as mean \pm SEM; unpaired Student's *t*-test; * $P < 0.05$ vs. Fas wt; # $P < 0.05$ vs. vehicle; § $P < 0.05$ vs. Fas wt/sTNF α -R

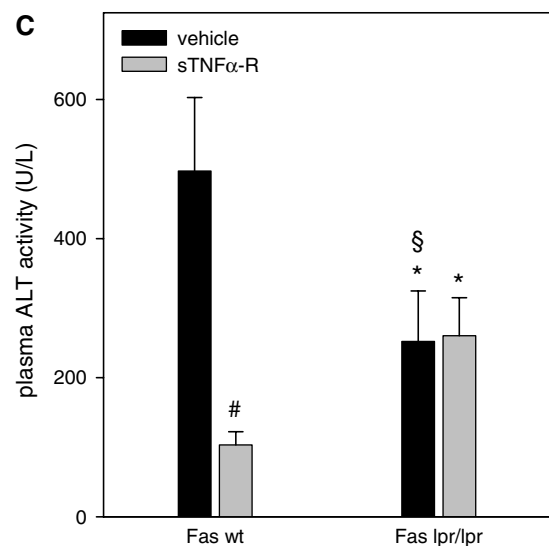
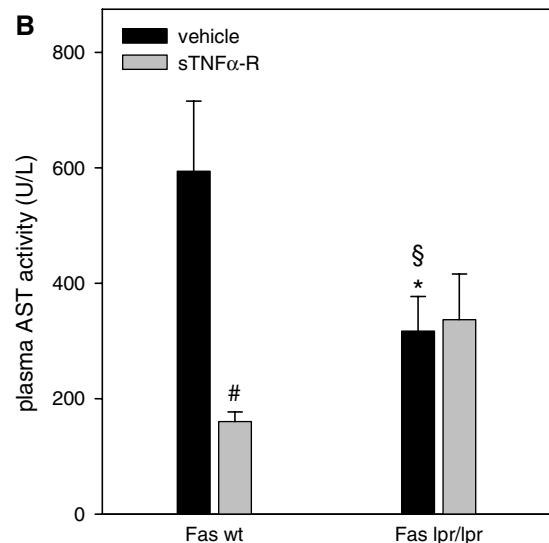
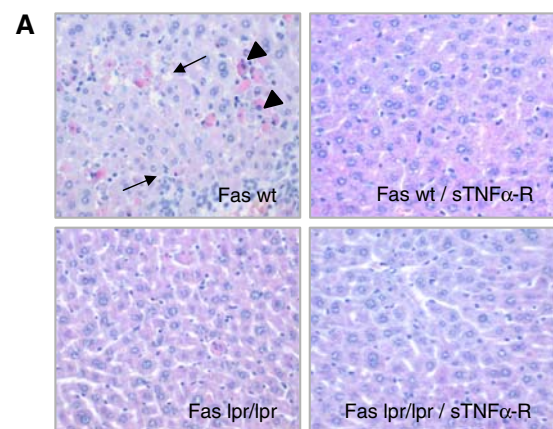
Western blot analysis in samples of whole-liver tissue. Referring to this, real-time RT-PCR quantification of Fas and FasL in whole-liver could exclude contamination with infiltrating cells based on the absence of CD3 cDNA signals [12].

Mediation of hepatocyte apoptosis in Gal/LPS-exposed livers

Apoptosis of hepatocytes is known to be a major cause of hepatocellular injury in a variety of liver diseases. Neutralization of TNF α by either monoclonal antibodies, inhibitors of TNF α processing, or anti-TNF α pharmacologic agents suppresses mortality in liver injury [25]. Similarly, it has been reported that TNF α plays a crucial role in the lethal activity of Gal/LPS [26] and that it is a terminal mediator for it [27]. The evidence that LPS-induced apoptosis in Gal-sensitized mice is mediated by TNF α release [8, 27] is further substantiated by the fact that anti-TNF α antibody [8] and sTNF α -R (present study) almost completely prevented LPS-induced hepatocyte apoptosis in Gal-exposed mice. Moreover, apoptosis of hepatocytes can be reproduced by injecting recombinant TNF α [8, 17].

Furthermore, it is well known that LPS induces TNF α release by Kupffer cells in the presence of functioning TLR-4 [28]. It has additionally been shown in vivo that activation of Kupffer cells and sinusoidal endothelial cells upon acute endotoxemia is largely dependent on TLR-4 [29]. In line with this, livers from Fas wt animals subjected to ALF showed a 2-fold rise in TLR-4 expression [30]. The observation from our current study that in Gal/LPS-challenged Fas lpr mutant mice, TLR-4 expression is markedly suppressed strongly indicates that interruption of Fas ligation modulates TLR-4 signalling [30], thereby contributing to the prevention of liver injury.

In addition to TNF α and its receptors, which signal apoptosis via an intracellular suicide cascade, ligation of cell-membrane Fas also triggers apoptosis in many cell types, including hepatocytes [31]. Although Fas has been implicated in a variety of chronic liver diseases that are



characterized by hepatocellular apoptosis [4, 13, 32–34], controversy exists regarding the contribution of Fas/FasL to inflammation-induced programmed cell death. Previous reports that neither Fas antigen-negative MRL/lpr nor

FasL mutant mice were protected against Gal/LPS-induced hepatic injury [6, 8] are in contrast with the findings of the present study, in which Fas lpr mutation conferred significant protection, as shown by the diminution in the number of apoptotic hepatocytes and the decreased activation of the downstream effector caspase-3. Caspase-3 is reported to be essential for Fas-mediated apoptosis in vitro [35]. An in vivo study could further demonstrate that caspase-3 is required for initial events that occur in hepatocyte cell death following Fas ligation [36]. Along with cleaved caspase-3 positive hepatocytes, we demonstrated a Gal/LPS-induced increase in Fas and FasL levels by use of immunohistochemistry, RT-PCR, and Western blot analysis, which was far less pronounced when TNF α was blocked. These findings strongly indicate that TNF α -induced activation of the Fas/FasL pathway may be a critical factor in the amplification of hepatocyte apoptosis, because FasL-expressing hepatocytes can cause Fas-mediated suicide or fratricide [13, 33]. This view is further underlined by the inhibition of TNF α -induced apoptosis of HepG2 cells by a neutralizing anti-FasL antibody. Furthermore, we determined the extent of apoptosis in HepG2 cells which have been cultured at high density. The tight cell-cell-contact might increase the degree of apoptosis in comparison to cells cultured at lower density (data not shown). In addition, Fas positive hepatocytes were found in the direct neighbourhood of hepatocytes which were FasL positive, therefore underscoring the fratricidal cell death by Fas-FasL interaction. Although we could not completely eliminate the possibility that inflammatory cells induce Fas/FasL-mediated hepatocellular apoptosis, we would like to conclude that particularly fratricidal death by Fas-FasL interactions of neighbouring hepatocytes may actively contribute to ALF.

Recently, it was shown that Fas ligation not only promotes apoptosis but also stimulates proinflammatory events such as macrophage infiltration and leukocyte recruitment during liver injury [37]. Leukocyte infiltration has been shown to be not only the cause, but also the consequence of hepatocyte apoptosis [38]. Thus, the marked inhibition of hepatocellular apoptosis in the Fas lpr mice might be due—at least in part—to a reduction in inflammatory leukocyte infiltration.

In conclusion, TNF α hepatotoxicity in Gal/LPS-induced acute liver failure involves Fas/FasL-dependent cell death in either a fratricidal or suicidal manner. Thus, when developing anti-apoptotic strategies, hepatocytes should be considered to be active contributors to apoptotic cell death.

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