Role of the perforin/granzyme cell death pathway in D-Gal/LPS-induced inflammatory liver injury

Angela Kuhla¹, Christian Eipel¹, Kerstin Abshagen¹, Nikolai Siebert¹, Michael D Menger², Brigitte Vollmar¹

¹Institute for Experimental Surgery, University of Rostock, Rostock, Germany
²Institute for Clinical & Experimental Surgery, University of Saarland, Homburg/Saar, Germany

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contact information:

Prof. Dr. med. B. Vollmar
Institute for Experimental Surgery
University of Rostock
Schillingallee 69a
D-18055 Rostock
Germany
phone: +49-381-494-6220
fax:+49-381-494-6222
e-mail: brigitte.vollmar@med.uni-rostock.de
Abstract

Cytotoxic T lymphocytes and their granule components, such as perforin and granzyme, play an important role in the defense of hepatic infections caused by different pathogens. Moreover, it has been shown in vitro that hepatocytes can initiate cell death via a perforin-dependent mechanism. Although it is well known that hepatocellular apoptosis in D-galactosamine/lipopolysaccharide (D-Gal/LPS)-associated liver failure is mediated by TNFα-dependent Fas/FasL cytotoxicity, there is no information on the role of perforin-mediated mechanisms in vivo. Therefore, we studied whether the cytolytic perforin/granzyme pathway contributes to the D-Gal/LPS-associated hepatotoxicity. Perforin knock out (Pko) mice showed significantly higher hepatic TNFα and IL-6 mRNA expression as well as plasma TNFα and IL-6 concentrations within the first hour upon D-Gal/LPS challenge when compared to perforin wild type (Pwt) mice. At 6 hours upon D-Gal/LPS challenge Pko mice further presented with higher transaminase release and onconecrotic tissue damage, while hepatocellular apoptosis and caspase-3 cleavage remained unaffected by the perforin deficiency. Pretreatment with a recombinant human TNFα-receptor fusion protein attenuated necrotic and apoptotic tissue damage, and reduced plasma transaminase activities as well as cytokine release, thereby preventing acute liver failure in Pko mice as effective as in Pwt mice.

These data do not only confirm the significance of TNFα as distal mediator of hepatic injury in this model, but simultaneously reveal a contribution of a perforin-dependent immunoregulation, limiting the D-Gal/LPS-induced overwhelming cytokine release and onconecrotic tissue injury.
Introduction

The liver provides the first line of defence against bacteria and bacterial products, such as lipopolysaccharide (LPS), crossing the intestinal barrier and, thus, plays a pivotal role in the innate immune response. To successfully combat bacterial infection, a sufficient inflammatory response is a prerequisite. However, there is evidence that self-inflicted damage to the liver as a consequence of an overtly aggressive inflammatory response is the cause for inflammatory liver disease which may result in acute liver failure and fatal patient outcome.

Although mechanisms of LPS-induced liver injury are not completely understood, it is well known that hepatocytes are the major target cells in acute liver failure and that inflammatory cells, such as macrophages and neutrophils, dominate the manifestation of injury (4, 5, 27). These initial innate responses have been discovered to be dependent on intact adaptive immune cells, which are shown to temper the ongoing early response to infection and to function as negative regulators (14). In line with this, it has been shown that CD4$^+$ lymphocytes, which are recruited in postischemic livers, suppress the oxidative burst activity of recruited neutrophils (3). At the same time, however, T-lymphocytes have been accused as key regulators in initiating postischemic inflammation in the liver, as tissue damage was dramatically reduced in T-cell deficient nu/nu mice (42).

In general, T-cells can exert cytotoxicity via exocytosis of their cytotoxic granules (26, 37, 41). The most important components of cytotoxic granules are the pore-forming protein perforin and the lymphocyte-specific granule serine esterase granzyme B (GrB) (24, 32, 33). Perforin and granzymes induce target-cell apoptosis cooperatively. Granzymes trigger caspase activation directly by caspase processing and indirectly by upregulation of pro-apoptotic genes, while perforin supports the appropriate delivery of granzymes by inducing transmembrane pores (37).

Previous studies with mouse strains deficient in one or more components of the granule-exocytosis pathway indicate that a concerted action of perforin and GrB is essential for the recovery from infections by pathogens, such as ectromelia virus (22), mouse
cytomegalovirus (28), herpes simplex virus (25), and Trypanosoma cruzi (23). However, prevention of pathology, liver disease and early host death during infection with T. cruzi has been found critically dependent in addition on an operational Fas/FasL interaction (23). Further, it has been shown in adenoviral construct-induced liver infection with T-cell function that the perforin-pathway of lytic activity is not demonstrable in vivo, while Fas- or TNFR1-dependent death receptor pathways are most effective in hepatocyte lysis (1, 21).

In the lethal model of inflammatory liver disease upon LPS exposure of D-Gal-sensitized mice, TNFα has repeatedly been demonstrated to predominantly mediate liver injury not only via its receptor TNFR1 (7), but also by concomitant activation of the Fas/FasL pathway (16). In line with this, it has been noticed that hepatocytes might actively contribute to liver injury by elimination of Fas-sensitive target cells (16). Apart from that, hepatocytes have been most recently shown to express biologically competent perforin capable of inducing death of contacted cells in vitro (8). This prompted us to assess the relevance of the perforin/granzyme cell death pathway in D-Gal/LPS-induced liver pathology of perforin wild type (Pwt) and perforin knock out (Pko) mice.
Material and Methods

Model of acute liver failure and experimental groups. Male perforin knock out mice (Pko; pfp^-/-; C57BL/6J; Jackson Laboratory, Maine, USA), generated as previously described (12), and perforin wild type mice (Pwt; C57BL/6J; Charles River Laboratories, Sulzfeld, Germany) were used at the age of 8 to 10 weeks with a body weight (bw) of approximately 20 g. Animals were kept on water and standard laboratory chow ad libitum. The experimental protocol was approved by the local animal care 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 acute liver failure, mice (n=26) were injected with D-Gal (720 mg/kg bw ip; Sigma-Aldrich, Taufkirchen, Germany) and LPS (10 µg/kg bw ip, E. coli serotype 0128:B12; Sigma-Aldrich) (5, 17). Additional series of time-matched sham-treated animals with application of equivalent volumes of 0.9% saline were performed and designated as controls (n=26).

To verify the contribution of TNFα in D-Gal/LPS-induced acute liver failure, additional animals were pretreated with sTNFα-R (recombinant human TNFR Fc fusion protein, etanercept, 100 µg/kg bw ip, Enbrel®; Wyeth Pharma GmbH, Münster, Germany) or equivalent volumes of 0.9% saline 1 h prior to exposure to D-Gal/LPS. Though being of human origin, etanercept (sTNFα-R) has been described as TNFα-blocking drug, preventing rheumatoid arthritis (40) and D-Gal/LPS-induced liver failure in mice (16).

To address the role of Kupffer cells, additional animals were applied Cl2MDP-liposomes (0.1 mL/10 g body wt) via the tail vein under light ether anesthesia 24 h before D-Gal/LPS exposure (2).

After 30 min, 1 h or 6 h of D-Gal/LPS exposure, animals were exsanguinated by puncture of the vena cava inferior for immediate separation of plasma, followed by harvest of liver tissue.

Sampling and assays. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were measured spectrophotometrically as indicators for
hepatocellular disintegration and necrosis. Plasma concentrations of TNFα and interleukin-6 (IL-6) were analyzed with a commercially available enzyme-linked immunosorbent assay kit in accordance with the manufacturer’s instructions (Pierce, Biotechnology, Rockford, IL, USA). Liver tissue was preserved for subsequent RNA and Western blot analysis as well as histology and immunohistochemistry.

**Analysis of TNFα and IL-6 mRNA expression in liver tissue by semiquantitative PCR.** Total RNA was isolated from liver tissue using a RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer’s instructions. RNA concentration was determined spectrophotometrically. First strand cDNA was synthesized by reverse transcription of 2 µg of total RNA using oligo(dT)18 primer (Biolabs, Frankfurt a. M., Germany) and Superscript II RNaseH-Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) in the presence of dNTP’s, 5 x first strand buffer and dithiothreitol at 72°C for 10 min and 42°C for 60 min. The reverse transcriptase was inactivated by 95°C for 5 min.

PCR for murine TNFα, IL-6 and GAPDH was performed in a 20 µl reaction volume containing 1x reaction puffer, 20 µM dNTPs, and 5000 U/mL Taq polymerase. Samples were incubated with respective primers (table 1) for either 30 (GAPDH), 32 (TNFα) or 33 (IL-6) cycles (denaturation for 30 s at 94 °C; annealing for 30 s at 59 °C (TNFα), 60 °C (IL-6), 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 (peqGOLD Universal Agarose; peQLab Biotechnologie, Erlangen, Germany) containing 0.5 µg/ml ethidium bromide. Signals were densitometrically assessed (Quantity One, ChemiDoc XRS System; Bio-Rad Laboratories, Munich, Germany) and normalized to the GAPDH signals.

**Analysis of perforin mRNA expression in liver tissue by real-time PCR.** Total RNA was isolated and cDNA was synthesized as described above. Expression levels of murine perforin mRNA were quantified using LightCycler System 1.5 (Roche, Manheim, Germany) relative to control samples and normalized to the expression of a housekeeping gene. For this approach we used SYBR Green I (Roche) for the detection of dsDNA amplified in the
PCR. The data represent expression of perforin gene product in relation to that of GAPDH as a housekeeping gene. Primers designed for analyzing gene expression amplify a fragment of 162 bp for perforin and 191 bp for GAPDH, spanning two exons to eliminate false positive signals from contaminating genomic DNA. Primers (table 1) were designed to anneal at about 55°C to combine both reactions in one run. The real-time PCR program included a 10 min denaturation step followed by 40 amplification cycles. The fluorescence intensity of the double-strand specific SYBR Green I, reflecting the amount of formed PCR product, was assessed at the end of each elongation step. After each LightCycler run a melting curve analysis was performed to analyze the products of the PCR. LightCycler PCR products were separated by 1% agarose (peqGOLD Universal Agarose; peQLab Biotechnologie, Erlangen, Germany) gel electrophoresis containing 0.5 µg/ml ethidium bromide once to confirm the correct amplification products. A standard curve was performed for perforin and GAPDH by dilution of cDNA to determine the efficiency of the target and the reference reaction. The cDNA for these dilution curves was generated from livers of five non-treated healthy animals. Both standard curve files were used to create a coefficient file that was used to correct for the different efficiency of the perforin and the GAPDH reaction. Furthermore, we used a calibrator (a standard sample) to compare the typical proportions of the target and the reference sequences to those of the samples. For the quantification of gene expression, duplicates were run in the LightCycler for both genes. The reaction without cDNA template was also performed as a negative control.

**Western blot analysis of liver tissue.** For Western blot analysis of protein levels of cleaved caspase-3, granzyme B (GrB), cation-dependent mannose-6 phosphate receptor (CD-MPR), and β-actin 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 and 0.2 mM PMSF, protease inhibitor cocktail), incubated for 30 min on ice and centrifuged for 15 min at 10,000 x g. Protein content were assayed by bicinchoninic acid (BCA) method (Pierce, Biotechnology) with bovine serum albumin (BSA) (Pierce, Biotechnology) as standard. Per lane 60 µg protein (cleaved caspase-3) and 20 µg protein (GrB, CD-MPR) was separated on a 12% SDS gel
and transferred to a polyvinylidifluoride membrane (Immobilon-P; Millipore, Eschborn, Germany). After blockade with 2% BSA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), membranes were incubated for 2 h at room temperature with a rabbit polyclonal anti-cleaved caspase-3-antibody (1:1,000; Asp 175; Stressgen Biotech, San Diego, CA, USA), a goat polyclonal anti-CD-MPR antibody (1:500; D-19; Santa Cruz Biotechnology) and a rabbit polyclonal anti-GrB antibody (1:10,000; Cell Signaling Technology, Boston, MA, USA), followed by a secondary peroxidase-linked goat anti-rabbit antibody (cleaved caspase-3; 1:2,000 and GrB; 1:20,000; Cell Signaling Technology) or a donkey anti-goat antibody (CD-MPR; 1:5,000; Santa Cruz Biotechnology). Protein expression was visualized by means of luminal-enhanced chemiluminescence (ECL plus; Amersham Pharmacia Biotech, Freiburg, Germany) and digitalized with ChemiDoc™ XRS System (Bio-Rad Laboratories, Munich, Germany). Signals were densitometrically assessed (Quantity One; Bio-Rad Laboratories) and normalized to the β-actin signals (mouse monoclonal anti-β-actin antibody; 1:20,000; Sigma).

Histology and immunohistochemistry of liver tissue. For H&E staining and immunohistochemical analysis of cleaved caspase-3- and TUNEL-positive hepatocytes, we used standard protocols as previously published by our group (5, 17, 30).

For immunohistochemical analysis of perforin and F4/80 positive liver macrophages, liver tissue was fixed in 4% phosphate-buffered formalin for 2-3 days and then embedded in paraffin. From the paraffin-embedded tissue blocks, 4 µm sections were put on poly-L-lysine covered glass slides and exposed to a rabbit polyclonal anti-mouse perforin (1:500; Cell Signaling Technology) or a rat monoclonal anti-mouse F4/80 antibody (1:10; Serotec, Oxford, UK). Universal LSAB® kits (System-HRP; DakoCytomation, Dako, Hamburg, Germany) were used according to the manufacturer’s instructions for the development of perforin with DAB chromogen. The sections were counterstained with hemalaun and analyzed with a light microscope (Olympus BX51, Hamburg, Germany). An alkaline phosphatase conjugated secondary goat anti-rat IgG antibody (1:200; Santa Cruz
Biotechnology) was used for the development of the F4/80 antigen. Positive cells were counted within 50 consecutive fields and given as cells per mm².

Granulocytes were stained with the AS-D chloroacetate esterase (CAE) technique and were identified by positive staining and morphology. CAE-positive cells were counted within 50 consecutive fields of liver tissue and are given as cells per mm².

Statistical analysis. All data are expressed as means ± SEM. Statistical differences were determined using ANOVA, followed by post-hoc pairwise comparison tests for analysis between either strains or treatment. Data were considered significant if *p*<0.05. Statistical analysis was performed using the SigmaStat software package (Jandel Scientific, San Rafael, CA, USA).

Results

Hepatocellular disintegration and necrosis. H&E histopathology of D-Gal/LPS-exposed livers exhibited a disruption of liver cell architecture and microvascular disintegration, accompanied by hepatocellular cell death and hepatic transaminase release (fig. 1 A-C). The extent of D-Gal/LPS-associated onconecrotic tissue damage was found markedly more pronounced in Pko mice when compared to Pwt controls (fig. 1 A). This was associated with significantly higher AST and ALT plasma activities (fig. 1, B and C).

Analysis of mRNA expression in liver tissue at 1 h and 6 h of D-Gal/LPS exposure demonstrated significantly higher transcript levels of hepatic TNFα (fig. 2 A, upper panel) and IL-6 (fig. 2 B, upper panel) in the Pko mice when compared to the Pwt mice. Concomitantly, the analysis of circulating TNFα (fig. 2 A, lower panel) and IL-6 (fig. 2 B, lower panel) showed notably higher concentrations in the D-Gal/LPS-challenged Pko mice. In case of depletion of hepatic macrophages by CldoMDP-liposomes, Pko animals demonstrated a reduction of liver injury and hepatocellular apoptosis, as indicated by a 2-fold decrease of transaminase levels and cleaved caspase-3 protein. In addition, macrophage-depleted Pko mice revealed significantly lower transcript levels of hepatic TNFα and IL-6 and plasma concentrations of
circulating TNFα and IL-6 (data not shown). These data underline the hepatic macrophages as main cellular source of cytokine release upon D-Gal/LPS challenge.

Though Pwt and Pko mice markedly differed with respect to cytokine production, the number of liver tissue macrophages was not different between both strains, neither under control conditions nor upon D-Gal/LPS exposure (fig. 3 A). In contrast, D-Gal/LPS-challenged livers demonstrated marked leukocytic tissue infiltration, as indicated by a more than 10-fold increase of CAE positive cells/mm² in livers of both Pwt and Pko mice compared to sham-treated controls (fig. 3 B).

**Hepatic perforin and granzyme B expression.** D-Gal/LPS challenge did not affect hepatic expression of perforin mRNA, neither in Pko nor in Pwt animals (fig. 4). However, perforin mRNA transcript levels in livers of Pko mice were found ~70% lower when compared to that of Pwt mice livers (fig. 4).

Analysis of perforin protein by immunohistochemical staining revealed a few positive hepatocytes in livers of sham-treated Pwt mice (fig. 5, upper left panel), while perforin protein was not detectable in D-Gal/LPS-exposed Pwt livers (fig. 5, upper right panel). Livers of Pko mice did not stain for perforin protein, neither under control conditions nor after D-Gal/LPS exposure (fig. 5, lower panel). Protein expression of GrB and its receptor CD-MPR was found decreased in both D-Gal/LPS-challenged Pwt (~30%) and Pko mice (~40%) compared to untreated controls (data not shown).

**Hepatocellular apoptosis.** Analysis of hepatocellular apoptosis is given in figures 6 and 7, displaying the data from TUNEL assay (fig. 6) and cleaved caspase-3 protein analysis (fig. 6). After D-Gal/LPS exposure, livers of both Pwt and Pko mice exhibited severe tissue damage with a high number of TUNEL positive cells when compared to livers of sham-treated controls (fig. 6). In line with this, immunohistochemical examination revealed high numbers of cleaved caspase-3 positive hepatocytes in livers of both Pwt and Pko mice after D-Gal/LPS challenge (fig. 7 A). Furthermore, Western blot analysis of the large fragment (17/19 kDa) of the activated caspase-3 confirmed the comparable amount of hepatocellular
apoptosis, as given by almost equal cleaved caspase-3 protein in both mouse strains (fig. 7 B).

**TNFα blockade in D-Gal/LPS-exposed Pwt and Pko mice.** In accordance to previous studies, describing a fundamental role of the TNFα pathway in mediating LPS-induced inflammatory liver disease (9, 16, 31, 38), pre-treatment of mice with sTNFα-R before the D-Gal/LPS challenge caused an almost complete abrogation of hepatocellular apoptosis and necrosis as well as a decrease in transaminase levels, being in Pko mice as effective as in Pwt mice (table 2).

**Discussion**

The present in vivo study indicates that the deletion of the perforin/granzyme cell death pathway does not affect leukocytic tissue infiltration and hepatocellular apoptosis in D-Gal/LPS-exposed livers, but results in an increase of onconecrotic cell death. The significantly higher hepatic mRNA expression and plasma concentrations of TNFα and IL-6 in D-Gal/LPS-challenged Pko mice suggest a perforin-dependent immunoregulation, limiting cytokine release and overwhelming tissue injury. The almost complete abrogation of both apoptotic and necrotic cell damage by TNFα blockade underlines the predominant role of this death receptor signaling pathway over perforin in mediating D-Gal/LPS-induced liver failure.

Perforin mRNA transcripts were still detectable in Pko mice, though far less in extent compared to Pwt mice. This is due to the fact that Pko mice were generated by insertion of a neo cassette into the perforin-encoding gene without deletion of the coding sequence. In consequence, the transcription is not suppressed from the perforin promoter up to the neo cassette in these mice (12). Of interest, D-Gal/LPS exposure did not affect hepatic levels of perforin mRNA, which is in line with the data of others, studying the effect of TNFα on hepatocellular perforin mRNA expression (8).

There is major body of evidence that cytotoxic effector mechanisms in clearance of viral infections exhibit large differences between organs. While perforin-deficient mice show readily apparent delays in clearance of cytomegalovirus from spleen (36) and salivary gland
clearance of murine cytomegalovirus (36) and adenoviral infection (1, 21) from the liver seems to be perforin-independent. Moreover, among the various effector pathways, Fas/FasL-mediated cytotoxicity plays a more prominent role compared to TNF/TNFR1 signaling in clearance of hepatic viral infections (10, 11). Distinct analysis of perforin-deficient T-lymphocytes confirmed that their efficacy to induce target cell apoptosis is reduced, however, not to that extent as plasma membrane lysis (15, 19), indicating that other, perforin-independent mechanisms are operational which cause apoptosis. In line with this, D-Gal/LPS-exposed livers of both Pwt and Pko mice revealed no differences in the amount of hepatocellular apoptosis.

Upon stimulation of D-Gal-sensitized mice with LPS, hepatic macrophages secrete pro-inflammatory cytokines, including IL-1, IL-6 and TNFα. TNFα induces hepatocellular apoptosis which has been identified as an early and possibly causal event in D-Gal/LPS-induced liver failure (16). This is followed by TNFα-mediated leukocyte infiltration and hepatocyte necrosis (13). Astonishingly, almost all parameters, i.e. numbers of F4/80-positive macrophages, infiltrating leukocytes and apoptotic hepatocytes were found comparably affected in Pko and Pwt mice upon D-Gal/LPS challenge. In fact, animals only differed in TNFα and IL-6 transcription as well as TNFα and IL-6 release, onconecrotic tissue damage and transaminase activities. For perforin-deficient cytotoxic lymphocytes, an overproduction of pro-inflammatory cytokines is described (29). In line with this, cytotoxic lymphocytes of children with the autosomal recessive disorder hemophagocytic lymphohistiocytosis, a syndrome of perforin deficiency, are unable to downregulate the immune response with the result of uncontrolled activation and accumulation of macrophages and overproduction of pro-inflammatory cytokines (39). Thus, the excessive TNFα and IL-6 surge in the D-Gal/LPS-challenged Pko mice might reflect the immune dysregulation due to perforin deficiency, similarly as observed for patients with hemophagocytic lymphohistiocytosis (34). As a consequence, Pko mice might suffer from acute metabolic perturbation and ATP depletion. The diminution of energy directs tissue injury from the ATP-dependent apoptotic cell death towards passive necrotic cell rupture.
(20), which in turn might explain the obvious proneness of Pko mice to increased onconecrotic tissue damage. In rats, it has been shown that D-Gal causes apoptosis in the liver by activating caspase-3, which is released to the plasma by secondary necrosis, as indicated by the concomitant AST increase (35). Thus, death processes which start with common death signals sharing similar pathways, but culminate in either cell lysis (necrotic cell death) or programmed cell death (apoptosis), known as necrapoptosis (6, 18), seem to characterize the nature of cell death in D-Gal/LPS-exposed Pko mice.

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References


D-Galactosamine-induced hepatic injury is mediated by TNF-alpha and not by Fas ligand.  


Figure legends

**Figure 1:** Representative H&E stained images (A, original magnification x400) as well as plasma AST (B) and ALT activities (C) in Pwt (n=14) and Pko animals (n=14). Animals were injected with either saline (control; n=14) or D-Gal (720 mg/kg bw ip) and LPS (10 µg/kg bw ip) for induction of acute liver failure (D-Gal/LPS; n=14). Analysis was performed six hours thereafter. Note the marked onconecrotic injury and the increased AST and ALT activities in D-Gal/LPS-exposed Pwt, but in particular Pko mice. Values are given as means ± SEM; ANOVA, post-hoc pairwise comparison tests: # p < 0.05 vs. Pwt; * p<0.05 vs. corresponding control.

**Figure 2:** Representative PCR gels and quantitative analysis of TNFα (A) and IL-6 (B) mRNA expression as well as analysis of plasma TNFα (A) and IL-6 (B) concentrations in Pwt (n=16) and Pko (n=16) mice. TNFα and IL-6 mRNA signals were corrected with that of GAPDH. Animals were injected with either saline (control; n=10) or D-Gal (720 mg/kg bw ip) and LPS (10 µg/kg bw ip) for induction of acute liver failure (D-Gal/LPS; n=22). Analysis was performed 30 min, 1 hour or 6 hours thereafter. Values are given as means ± SEM; ANOVA, post-hoc pairwise comparison tests: # p < 0.05 vs. Pwt; * p<0.05 vs. corresponding control.

**Figure 3:** Representative light microscopic images (upper panels, original magnification x400) and quantitative analysis (lower panels) of F4/80 positive macrophages (A) and of CAE positive leukocytes (B) in livers of Pwt (n=14) and Pko animals (n=14). Animals were injected with either saline (control; n=14) or D-Gal (720 mg/kg bw ip) and LPS (10 µg/kg bw ip) for induction of acute liver failure (D-Gal/LPS; n=14). Analysis was performed six hours thereafter. Values are given as mean ± SEM. ANOVA, post-hoc pairwise comparison tests: * p<0.05 vs. corresponding control.
Figure 4: Representative real-time PCR SYBR Green I fluorescence curves, PCR gels and relative quantitative analysis of perforin mRNA levels in livers of Pwt (n=10) and Pko (n=10) mice. Perforin signals were corrected with that of GAPDH. Animals were injected with either saline (control; n=10) or D-Gal (720 mg/kg bw ip) and LPS (10 µg/kg bw ip) for induction of acute liver failure (D-Gal/LPS; n=10). Analysis was performed six hours thereafter. Values are given as means ± SEM; ANOVA, post-hoc pairwise comparison tests: # p < 0.05 vs. Pwt.

Figure 5: Representative light microscopic images (original magnification x400) of perforin immunohistochemistry in livers of Pwt and Pko animals. Animals were injected with either saline (control) or D-Gal (720 mg/kg bw ip) and LPS (10 µg/kg bw ip) for induction of acute liver failure (D-Gal/LPS). Note the few perforin-positive hepatocytes in sham-treated Pwt mice (arrow heads, upper left image).

Figure 6: Representative light microscopic images (upper panel, original magnification x400) and quantitative analysis (lower panel) of TUNEL assay for apoptotic hepatocytes in Pwt (n=14) and Pko animals (n=14). Animals were injected with either saline (control; n=14) or D-Gal (720 mg/kg bw ip) and LPS (10 µg/kg bw ip) for induction of acute liver failure (D-Gal/LPS; n=14). Analysis was performed six hours thereafter. Note the high numbers of TUNEL-positive hepatocytes after D-Gal/LPS exposure in both Pwt and Pko mice (upper panel). Values are given as means ± SEM; ANOVA, post-hoc pairwise comparison tests: * p<0.05 vs. corresponding control.

Figure 7: Representative light microscopic images of cleaved caspase-3 immunohistochemistry (A, original magnification x400) as well as Western blot and densitometric analysis of cleaved caspase-3 protein levels (B) in livers of Pwt (n=14) and Pko animals (n=14). Cleaved caspase-3 signals were corrected with that of β-actin. Animals were injected with either saline (control; n=14) or D-Gal (720 mg/kg bw ip) and LPS (10 µg/kg bw ip) for induction of acute liver failure (D-Gal/LPS; n=14). Analysis was performed six hours
thereafter. Values are given as means ± SEM; ANOVA, post-hoc pairwise comparison tests:

* p<0.05 vs. corresponding control.
Table 1: List of primers used for semiquantitative and real-time PCR

<table>
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<th>Transcript</th>
<th>Forward primer (5′– 3′)</th>
<th>Reverse primer (5′– 3′)</th>
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<td>TNFα</td>
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Table 2: Hepatocellular apoptosis as well as transaminase and IL-6 release in Pwt and Pko mice at 6 h upon induction of acute liver failure by D-Gal/LPS. Animals were pretreated with sTNFα-R 1 h before the D-Gal/LPS exposure.

<table>
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<th>Parameters</th>
<th>Pwt D-Gal/LPS + sTNFα-R</th>
<th>Pko D-Gal/LPS + sTNFα-R</th>
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<td>TUNEL positive hepatocytes (n/mm²)</td>
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<td>3.4 ± 1.7*</td>
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<td>cleaved caspase-3 protein (rel. density)</td>
<td>0.2 ± 0.1*</td>
<td>0.5 ± 0.2*</td>
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<td>AST (U/mL)</td>
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<td>169 ± 23*</td>
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<tr>
<td>ALT (U/mL)</td>
<td>94 ± 16*</td>
<td>71 ± 3*</td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>687 ± 229</td>
<td>371 ± 84*</td>
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Values are given as mean ± SEM; unpaired Student’s t-test; * p<0.05 vs. D-Gal/LPS-treated animals without sTNFα-R (see corresponding figures).
Fig. 1
Fig. 2
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Fig. 4
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Fig. 6
Fig. 7