

Published in final edited form as:

J Hepatol. 2014 February ; 60(2): 298–305. doi:10.1016/j.jhep.2013.09.013.

A novel mouse model of depletion of stellate cells clarifies their role in ischemia/reperfusion- and endotoxin-induced acute liver injury

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Abstract

Background & Aims—Hepatic stellate cells (HSCs) that express glial fibrillary acidic protein (GFAP) are located between the sinusoidal endothelial cells and hepatocytes. HSCs are activated during liver injury and cause hepatic fibrosis by producing excessive extracellular matrix. HSCs also produce many growth factors, chemokines and cytokines, and thus may play an important role in acute liver injury. However, this function has not been clarified due to unavailability of a model in which HSCs are depleted from the normal liver.

Methods—We treated mice expressing HSV-thymidine kinase under the GFAP promoter (GFAP-Tg) with 3 consecutive (3 days apart) CCl₄ (0.16 µl/g; ip) injections to stimulate HSCs to enter the cell cycle and proliferate. This was followed by 10-day ganciclovir (40 µg/g/day; ip) treatment, which is expected to eliminate actively proliferating HSCs. Mice were then subjected to hepatic ischemia/reperfusion (I/R) or endotoxin treatment.

Results—CCl₄/ganciclovir treatment caused depletion of the majority of HSCs (about 64-72%), while the liver recovered from the initial CCl₄-induced injury (confirmed by histology, serum ALT and neutrophil infiltration). The magnitude of hepatic injury due to I/R or endotoxemia (determined by histopathology and serum ALT) was lower in HSC-depleted mice. Their hepatic

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Conflict of interest: None of the authors participated in this study have anything to disclose regarding conflict of interest.

expression of TNF- α , neutrophil chemoattractant CXCL1 and endothelin-A receptor also was significantly lower than the control mice.

Conclusions—HSCs play an important role both in I/R- and endotoxin-induced acute hepatocyte injury, with TNF- α and endothelin-1 as important mediators of these effects.

Keywords

Hepatic stellate cells; Depletion; Ischemia/Reperfusion; Endotoxemia; Lipopolysaccharide; Hepatocytes; Liver; Injury; Necrosis; Apoptosis

Introduction

The perisinusoidal hepatic stellate cells (HSCs) constitute 8-12% of the liver cell population, express glial fibrillary acidic protein (GFAP) and/or desmin, and are the major storage site of retinoids [1]. During liver injury, HSCs undergo activation characterized by the loss of retinoids, expression of α -smooth muscle actin and differentiation into proliferating myofibroblast-like cells. Activated HSCs produce excessive extracellular matrix (ECM) and exhibit increased expression of tissue inhibitors of metalloproteinases and reduced or unchanged expression of matrix metalloproteinases [2-4], thus becoming the major cell type responsible for hepatic fibrosis [5]. Activated HSCs are postulated to contribute to portal hypertension by their high contractility and up-regulation of the powerful vasoconstrictor endothelin-1 (ET-1) and its receptors [6,7].

HSCs express intercellular adhesion molecule-1 [8], produce various cytokines and chemokines [8-10], and thus can play important role in hepatic inflammation. Gram-negative bacterial endotoxin (lipopolysaccharide:LPS) stimulates the synthesis of nitric oxide (NO), ET-1, tumor necrosis factor (TNF)- α and interleukin (IL)-6 in both quiescent and activated HSCs; LPS-challenged HSCs stimulate NO synthesis, inhibit DNA synthesis and cause apoptosis of cultured hepatocytes [11-14].

Recent work demonstrates that HSCs also influence hepatic immunological functions. HSCs induce apoptosis of allogeneic CD4⁺ and CD8⁺ T cells [8,15], present bacterial lipid antigens to NKT cells [16], expand immunosuppressive regulatory T cells [8], and render dendritic cells immunosuppressive [10]. Furthermore, HSCs secrete powerful antioxidant protein(s) that protect hepatocytes from ischemia/reperfusion (I/R) injury [17].

Thus, HSCs are highly versatile cells that can profoundly influence hepatic structure and functions in physiology and pathology. Most of the *in vivo* work confirming their role in hepatic pathology has been focused on fibrosis. A fungal metabolite gliotoxin was found to cause apoptosis of activated rat and human HSCs *in vitro*, and of rat HSCs *in vivo* resulting in resolution of fibrosis [18,19]. However, gliotoxin also induces apoptosis of KCs and endothelial cells in the fibrotic liver [20,21]. Ebrahimkhani et al [22] administered gliotoxin into bile duct-ligated mice in conjugation with single-chain antibody C1-3, which recognizes synaptophysin expressed by activated HSCs [23]; C1-3-gliotoxin caused resolution of fibrosis by selectively depleting HSCs. Using a similar mouse model described in the present study, it was reported recently that concomitant treatment of B6.Cg-Tg(Gfap-

Tk)7.1Mvs/J transgenic mice with ganciclovir promoted depletion of HSCs, and caused amelioration of CCl₄-induced fibrosis and hepatic injury [24]. However, the role of HSCs in acute injury to the normal liver has not been evaluated. Here, we show amelioration of I/R- and endotoxin-induced acute injury to otherwise normal HSC-depleted liver, suggesting HSCs' critical role in pathologies unrelated to activation-dependence.

Materials and methods

Animals

The protocols were approved by the IACUC according to NIH guidelines. Wild-type male C57BL/6 (WT-B6) and B6.Cg-Tg(Gfap-Tk)7.1Mvs/J (GFAP-Tg) mice were from The Jackson laboratory. GFAP-Tg mice express the herpes simplex virus thymidine kinase (HSV-TK) transgene under the GFAP promoter [25]. HSV-TK phosphorylates nontoxic ganciclovir (GCV) to GCV-monophosphate, which is converted to GCV-triphosphate by cellular guanylate kinase; phosphorylated GCV incorporates into the DNA causing death of replicating cells [25,26]. GFAP is expressed exclusively by HSCs in the liver, which are quiescent physiologically [1]. We treated the GFAP-Tg mice or WT-B6 mice with three CCl₄ injections (0.16 µl/g in 50 µl peanut oil; ip), 3 days apart; control mice received peanut oil. After the last CCl₄ injection, each group was divided into two subgroups, one receiving GCV (40 µg/g/day; ip) and the other vehicle (PBS) for 10 days. CCl₄ treatment activates HSCs, which enter the cell cycle thus making them susceptible to phosphorylated GCV-induced death. Mice were subjected to I/R or endotoxin the day after completion of GCV treatment (Supplementary Fig. 1).

Ischemia/Reperfusion (I/R) or endotoxemia

For I/R, all structures (portal vein, hepatic artery and bile duct) to the left liver lobes were occluded with a microvascular clamp for 60 min. The right lobes served as control. The clamps were removed and the abdominal incision closed. For endotoxemia induction, mice were administered LPS (10 mg/kg) intraperitoneally. Blood was drawn at 6h following reperfusion or LPS administration for serum enzyme measurement. The livers were excised, washed in ice-cold PBS and portions were fixed in 10% buffered formalin or paraformaldehyde, or snap-frozen in liquid nitrogen.

Histological determinations

The sections of formalin-fixed tissue were stained with hematoxylin and eosin (H/E) for histopathological examination, with TUNEL-stain (ApopTag Peroxidase kit, Chemicon) to detect apoptotic cells, or immunostained using rat-anti-mouse F4/80 Ab (Serotec), biotinylated goat-anti-rat secondary Ab (Jackson Immunoresearch) and ABC Elite kit (Vector Laboratories) to detect KCs.

The sections of paraformaldehyde-fixed frozen tissue were immunostained with anti-desmin rabbit polyclonal Ab (Abcam), anti-GFAP rabbit polyclonal Ab (DakoCytomation), rat anti-mouse F4/80 Ab (BioLegend) or rat anti-mouse TNF-α Ab (R&D Systems) as described previously [8, 10].

Neutrophils were identified immunohistochemically using Naphthol As-D Chloroacetate Esterase Kit (Sigma-Aldrich). Neutrophil accumulation was quantified in at least 4 randomly selected high-power fields (400X) of each liver section.

mRNA analysis

RNA was prepared from the snap-frozen tissue using TRIzol Reagent (Invitrogen), and cDNA was prepared using high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative real-time-PCR (qRT-PCR) was performed using the Sybr green master mix and 7500 Fast Real-Time PCR System (Applied Biosystems) with PCR primers listed in Table 1.

Statistics

Statistical significance was determined by non-parametric Kruskal-Wallis One-Way ANOVA on Ranks. A 'p' value <0.05 was considered statistically significant.

Results

Characterization of the CCl₄ effect on WT mice

To ensure that at the end of GCV treatment of mice, there is no residual hepatic injury due to earlier CCl₄ administration before subjecting them to I/R or endotoxemia, WT-B6 mice were sacrificed a day after the third CCl₄ injection or after 10 days of GCV treatment following termination of CCl₄. There was significant hepatic injury the day after third CCl₄ administration (Fig. 1A,B-middle panels), in both centrilobular and periportal areas (Supplementary Fig. 2), accompanied by inflammatory infiltration (Fig. 1B,C), and increased serum ALT (Fig. 1D). However, at the end of GCV treatment, liver histology and inflammation (Fig. 1A,B-lower panels; Fig. 1C) as well as serum ALT (Fig. 1D) had returned to normal and were identical to that in control mice.

Depletion of HSCs from the GFAP-Tg mice

Similar to WT-B6 mice (Fig. 1), no evidence of liver injury was observed in the GFAP-Tg mice after completion of CCl₄/GCV treatment as determined by histopathology and serum ALT (Supplementary Fig. 3A,B). GFAP immunostaining (Fig. 1E) demonstrated effective elimination of GFAP-positive cells from CCl₄/GCV-treated GFAP-Tg mice in periportal and centrilobular areas (64-72%; Supplementary Fig. 3C); qRT-PCR confirmed strong reduction in GFAP expression (~80%) (Fig. 1F). Several apoptotic cells were observed in the hepatic sinusoids of CCl₄/GCV-treated GFAP-Tg mice, but not in the CCl₄/PBS-treated mice (Fig. 1G). We performed desmin staining since nearly 80-90% of HSCs express both GFAP and desmin, while the remainder cells express either GFAP or desmin [1]. Thus, desmin⁺ but GFAP⁻ HSCs will not be eliminated due to GCV treatment. Indeed, there were significant desmin⁺ HSCs in CCl₄/GCV-treated GFAP-Tg liver, and no effect of these treatments was observed on KCs (Supplementary Fig. 4).

Morphological (not shown) and histopathological examination showed no apparent injury to other organs including intestine (Supplementary Fig. 5) or other gastrointestinal compartments indicating that GCV (40 µg/g/d:ip) treatment affected the liver selectively.

Amelioration I/R- and endotoxemia-induced injury in HSC-depleted mice

The mechanisms of hepatic I/R injury involve damage due to ischemia as well as reactive oxygen species (ROS) and inflammatory mediators produced by infiltrating monocytes and neutrophils, and KCs upon reperfusion [27,28]. HSCs, located in close association with hepatocytes, also produce ROS, proinflammatory mediators and chemokines [7-10], and thus may contribute to I/R injury. The production of the inflammatory mediators and chemokines by HSCs increases upon exposure to LPS [8,12-14]. Therefore, we investigated whether HSC-depletion affects warm I/R- or endotoxin-induced liver injury.

As expected, I/R caused profound parenchymal injury in the WT-B6 mice (Fig. 2A) with corresponding increase in serum ALT (Fig. 2B). The WT-B6 mice pretreated with CCl₄/GCV had normal liver architecture and serum ALT levels, and their liver injury due to I/R was similar to that in vehicle-treated WT-B6 mice. However, I/R injury to HSC-depleted mice was markedly reduced (Fig. 2A,B). I/R injury in B6-WT mice treated with CCl₄/PBS or oil/GCV, and GFAP-Tg mice treated with oil/PBS, CCl₄/PBS or oil/GCV demonstrated injury similar to that in B6-WT mice treated with oil/PBS (Supplementary Fig. 6A). Furthermore, the number of GFAP⁺ cells in all of these treatment groups was similar (Supplementary Figure 6B, 6C), and statistically not different from B6-WT mice treated with CCl₄/GCV. Neutrophil infiltration in the CCl₄/GCV-treated WT-B6 mouse I/R lobes was similar to that in the control I/R lobes, but was modestly reduced in the I/R lobes of HSC-depleted mice (Fig. 2C). The number of neutrophils in the non-ischemic lobes increased similarly in all treatment groups as compared to sham control liver (Fig. 2C).

TUNEL-staining demonstrated modest apoptosis of hepatocytes in vehicle and CCl₄/GCV-treated WT-B6 mice upon LPS administration (Fig. 3A,B). Hepatocyte apoptosis was significantly, but not completely, abrogated in HSC-depleted mice (Fig. 3A,B). These observations were consistent with similarly modest increase in serum ALT in vehicle- or CCl₄/GCV-pretreated WT-B6 mice upon LPS administration; endotoxin-induced injury was significantly lower in HSC-depleted GFAP-Tg mice (Fig. 3C). Furthermore, hepatic neutrophil infiltration was lower in LPS-administered HSC-depleted mice than in vehicle- or CCl₄/GCV-pretreated control mice (Fig. 3D). Interestingly, apoptotic cells were observed in the sinusoids of CCl₄/GCV-treated GFAP-Tg mouse liver (Fig. 3A-inset) indicating continued elimination of HSCs.

Expression of TNF- α , IL-6 and CXCL1

TNF- α and IL-6 are implicated in I/R- and LPS-induced hepatic injury. Hepatic mRNA expression of TNF- α , but not IL-6, increased significantly upon I/R in control mice (Fig. 4A). While the expression of TNF- α was lower in HSC-depleted I/R liver that of IL-6 increased significantly (Fig. 4A). LPS treatment increased TNF- α and IL-6 mRNA expression in control and HSC-depleted mice, and although this effect was reduced in the HSC-depleted mice, it was statistically significant for TNF- α and not IL-6 (Fig. 4B). Given the prominent role of TNF- α in I/R- and LPS-induced liver injury, we determined its protein expression immunohistochemically. The immunostaining for TNF- α increased strongly in the WT-B6 but not in HSC-depleted mice following I/R or endotoxemia (Supplementary Fig. 7). Hepatic mRNA expression of CXCL1, which plays an important role in neutrophil

chemotaxis in the injured tissue, increased in mice subjected to I/R as well as endotoxin treatment, but the increase was significantly lower in HSC-depleted livers (Fig. 4A,B).

Expression of ET-1 and its receptors

ET-1, through its vasoconstrictor activity, also contributes to I/R- [29,30] and LPS-induced [31] liver injury. The mRNA expression of ET-1 was not altered significantly upon I/R in control and HSC-depleted mice (Fig. 4C). ET-1 mRNA expression was up-regulated upon LPS treatment in control mice and, interestingly, increased further in LPS-treated HSC-depleted mice (Fig. 4D). ET-1 acts through two major receptors on various cell types: ET_A-R and ET_B-R. The direct effect of ET-1 on ET_A-R induces vasoconstriction while ET_B-R mediates vasodilation by stimulating release of nitric oxide and prostacyclin [32]. We found significant increase in ET_A-R expression in WT-B6 but not in HSC-depleted mice subjected to I/R or endotoxin (Fig. 4C,D). In contrast, ET_B-R expression increased similarly in both WT-B6 and HSC-depleted mice upon I/R or endotoxin treatment.

Discussion

The role of activated HSCs in hepatic fibrosis and cirrhosis has been established [5,33]. Recent research with cell culture studies and some indirect *in vivo* evidence indicate that quiescent or transiently activated HSCs (found in the liver during early phase of acute injury) can significantly influence metabolic and hemodynamic properties of the liver [7]. However, the lack of animal models in which HSCs are selectively depleted has been a major drawback to demonstrate unequivocally their precise contribution to acute liver injury. Herein, we demonstrate, for the first time, that HSCs play important role in I/R or endotoxin-induced hepatic injury using HSC-depleted mice.

Nearly 80% of the murine HSCs express GFAP [1], and thus the transgenic mouse in which HSV-TK is expressed under the GFAP promoter [25] provides a tool to generate an effective HSC-depleted model. However, in order for the toxic phosphorylated GCV to integrate into cellular DNA and induce cell death [34], the cells must leave their quiescent G0 state and enter the cell cycle. To achieve this, we treated GFAP-Tg mice with CCl₄ to induce proliferation of HSCs. The CCl₄-induced liver injury was repaired during the 10 days of GCV treatment while causing profound loss of HSCs. However, the liver was not completely devoid of HSCs as determined by desmin immunostaining, which may be due to the fact that about 10-15% of the cells do not express GFAP [1] and some GFAP⁺ cells may have escaped GCV-induced death. All of the other organs, including the gastrointestinal compartments were found to have no evidence of any injury. We note that GFAP-Tg mice that demonstrated severe inflammation and necrosis of the jejunum and ileum were treated continuously with 100 µg/g/d GCV [25], while we administered 40 µg/g/day GCV as bolus ip injections. We also note that bolus administrations of 100 µg/g/d GCV to GFAP-Tg mice did not elicit injury to other organs [24].

I/R injury is a complex phenomenon with inflammatory mediators and ROS produced by resident macrophages (KCs) and infiltrating blood cells such as neutrophils playing an important role. Several laboratories have observed that HSCs also produce the same mediators that can induce hepatocyte injury [7]. We have shown that LPS-stimulated HSCs

produce inflammatory mediators and chemokines [8,10,12-14], and induce apoptosis of hepatocytes [13]. With their close proximity to hepatocytes and number similar to KCs, we hypothesized that HSCs may play a significant role both in I/R and LPS-induced liver injury.

In contrast to the highly defined and extensive injury to untreated as well as CCl₄/GCV-pretreated WT mice, the I/R injury to HSC-depleted mice was diffuse and significantly reduced. Likewise, LPS-induced injury was also significantly reduced in HSC-depleted mice. Furthermore, neutrophil infiltration was lower in the I/R lobes and endotoxemic liver of HSC-depleted mice than in control mice suggesting that HSC-derived chemokines may contribute to their chemotaxis. Indeed, hepatic mRNA expression of a potent neutrophil chemoattractant CXCL1, which is produced by HSCs [8,10], was significantly lower in HSC-depleted mice. Both I/R- and endotoxemia-induced neutrophil infiltration also involve chemokines released by KCs [28,35], which might explain incomplete abrogation of neutrophil infiltration in HSC-depleted mice. Curiously, we observed significant, but similar in magnitude, neutrophil infiltration in the non-ischemic lobes of WT and HSC-depleted mice following reperfusion suggesting cross-trafficking of chemoattractants from the ischemic lobes.

It has been demonstrated that TNF- α causes injury to hepatocytes [36,37], while IL-6 protects them from I/R and acetaminophen-induced liver damage [38]. The smaller increase in TNF- α expression in HSC-depleted livers following I/R and endotoxin treatment suggests that HSCs might be major producers of TNF- α . Contrarily, IL-6 expression increased in HSC-depleted liver after I/R, and was similarly increased in LPS-treated control and HSC-depleted livers. Since LPS stimulates IL-6 synthesis in HSCs [8,12-14], our observations suggest that other cell types (e.g., KCs) may compensate for IL-6 production. Alternatively, it is plausible that HSCs might down-regulate IL-6 expression in other cells types. Nevertheless, increased IL-6 and reduced TNF- α in HSC-depleted liver appear to be a protective mechanism of I/R or endotoxemia-induced hepatic injury.

Amelioration of increased portal pressure in CCl₄-induced acute liver injury [39] and I/R [29,30] by antagonism by ET-1 action suggested that up-regulation of ET-1 and its receptors is a mechanism of this effect. LPS also up-regulates ET-1 expression in HSCs [11], and in the liver [31,40]. The role of HSCs in hepatic vasoconstriction is supported by the finding that ET-1 antagonism attenuates contraction of HSCs as well as increased portal pressure in experimental animals [31,41]. ET-1-induced contraction of quiescent (nontransformed) HSCs *in vivo* [42] and in cell culture [43] suggests that sinusoidal narrowing due to the action of ET-1 on HSCs may contribute to I/R- as well as endotoxin-induced liver injury. An important mechanism of increased hepatic ET-1 expression in I/R and endotoxemia could be the stimulatory effect of KC-derived TNF- α and/or TGF- β , which increase ET-1 release by endothelial cells and HSCs [44-46]. Blocking KCs activity by gadolinium chloride (GdCl₃) reduced ET-1 secretion and improved liver function after warm I/R in rats [47]. Furthermore, treatment with neutralizing TNF- α antibody ameliorated I/R injury [48,49], and carbon monoxide inhalation by recipient animals caused inhibition of hepatic graft TNF- α and IL-6 expression and reduced I/R injury [50]. LPS also stimulates production of TNF- α and IL-6 by KCs [51-54]. Together, these observations suggest that ET-1 contributes

to I/R as well as endotoxin-mediated liver damage. However, the results of this study show that the increase in ET-1 mRNA expression after I/R in WT mice was statistically not significant and did not change in the HSC-depleted mice. On the other hand, hepatic ET-1 mRNA increased further in HSC-depleted mice after endotoxin treatment. These data are paradoxical as LPS increases ET-1 expression in HSCs [11] and the whole liver [31]. A plausible interpretation of this finding is that LPS also induces counter-regulatory mechanisms that abrogate ET-1 expression. For example, synthesis of NO, which down-regulates ET-1 expression (55,56), is increased strongly in HSCs upon exposure to LPS and LPS-stimulated HSCs elicit a similar response in hepatocytes [11-14]. In the HSC-depleted liver, the loss of such mechanism(s) may be responsible for increased ET-1 expression in other cells (e.g., endothelial cells). Nevertheless, the results suggest that enhanced ET-1 release from the endogenous stores and/or up-regulation of ET-1 receptors during I/R and endotoxemia might be the major factors in hepatic injury. Indeed, whereas I/R and endotoxin increased ET_A-R and ET_B-R expression significantly in WT-B6 mice, only ET_A-R expression was significantly lower in HSC-depleted mice. These results suggest that ET-1-related (ET_A-R-mediated) contraction plays an important role in I/R and endotoxin-induced liver injury, and that this probably overrides the effect of vasodilatory mediators released in response to ET_B-R stimulation in the presence of HSCs.

While this work was in progress, depletion of HSCs during concomitant treatment with CCl₄ and GCV was reported to ameliorate fibrosis and the on-going CCl₄-induced hepatic injury in GFAP-Tg mice [24]. This is an interesting observation as hepatocytes metabolize CCl₄ (via cytochrome P450) to CCl₃, which then elicits cellular injury. Thus the findings of Puch et al [24] suggest that HSCs may contribute to CCl₄-induced hepatocyte death. In this regard, it was reported that GdCl₃-induced blockade of KCs reduced neutrophil infiltration and almost completely prevented CCl₄-induced hepatocyte death, without altering cytochrome P450-mediated CCl₄ metabolism [57]. Since HSCs also produce chemokines that induce neutrophil infiltration (Fig. 4) [8,10], a similar mechanism of reduced CCl₄-induced liver injury in HSC-depleted mice [24] is a likely possibility. Although the precise mechanisms of how HSCs mediate CCl₄-induced hepatic parenchymal damage [24] remain to be determined, our results indicate that HSCs are major participants of clinically relevant liver damage via inflammation, cytokines, chemokines and vasoactive mediators. Thus the blockade of the mechanisms by which HSCs induce acute liver injury has potential therapeutic relevance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ms. Rebecca B. Schuster for excellent technical assistance.

Financial Support: NIH PO1A1081678 and VA Merit Review Award 11O1BX001174, and Department of Surgery, University of Cincinnati and Children's Hospital Medical Center, Cincinnati.

Abbreviations

HSC	hepatic stellate cell
GFAP	glial fibrillary acidic protein
ECM	extracellular matrix
ET-1	endothelin-1
LPS	lipopolysaccharide
NO	nitric oxide
NOS	nitric oxide synthase
TNF	tumor necrosis factor
IL	interleukin
I/R	ischemia/reperfusion
KCs	Kupffer cells
WT	wild type
Tg	transgenic
HSV	herpes simplex virus
TK	thymidine kinase
GCV	ganciclovir
IP	intraperitoneal
CCl₄	carbon tetrachloride
PBS	phosphate-buffered saline
H/E	hematoxylin and eosin
Ab	antibody
qRT-PCR	quantitative real-time polymerase chain reaction
ALT	alanine aminotransferase
ROS	reactive oxygen species

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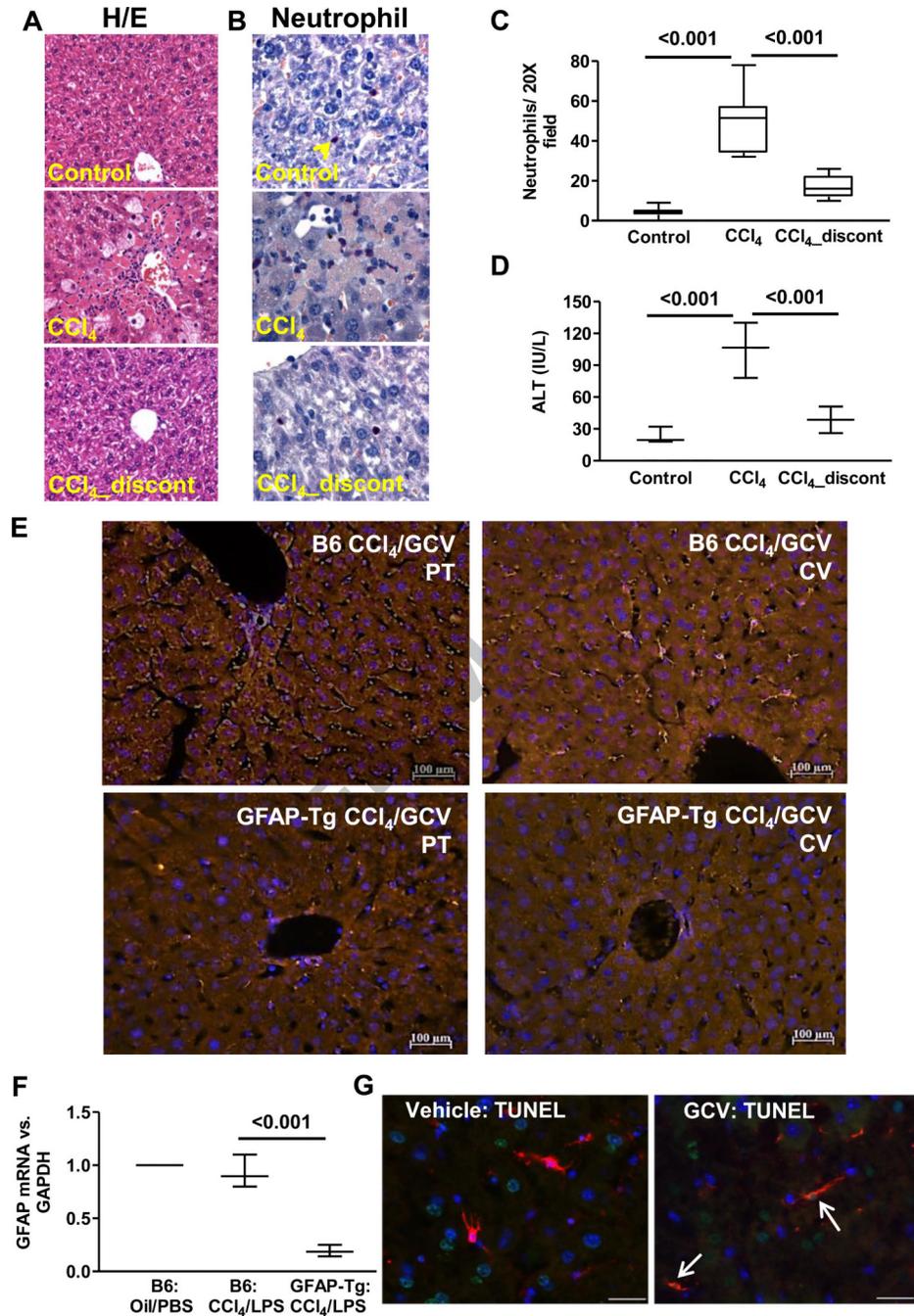


Fig. 1. Recovery of the liver from CCl4-induced injury

H/E staining (A), neutrophil staining (B) and quantification (C), and serum ALT levels (D) demonstrating liver-injury in WT-B6 mice (n=4) a day after 3 CCl4 injections, and its resolution on day 11 after termination of CCl4. Effect of CCl4/GCV treatment on HSC-depletion in GFAP-Tg mice. Mice received CCl4 (3 injections) followed by its discontinuation and GCV treatment of 10 days. (E) GFAP-immunostaining in livers showing depletion of HSCs from portal (PT) and centrilobular (CV) regions of the GFAP-Tg mice at the end of CCl4/GCV treatment. (F) Strong reduction in GFAP mRNA

expression in HSC-depleted mice. (G) TUNEL (green)- and GFAP (red)-stained cells are seen in CCl₄/GCV-treated GFAP-Tg mice and not in CCl₄/vehicle-treated mice at 2 days during vehicle or GCV treatment. Several apoptotic hepatocytes can also be seen at this time in both sections, which are replaced by day 10 after CCl₄ discontinuation.

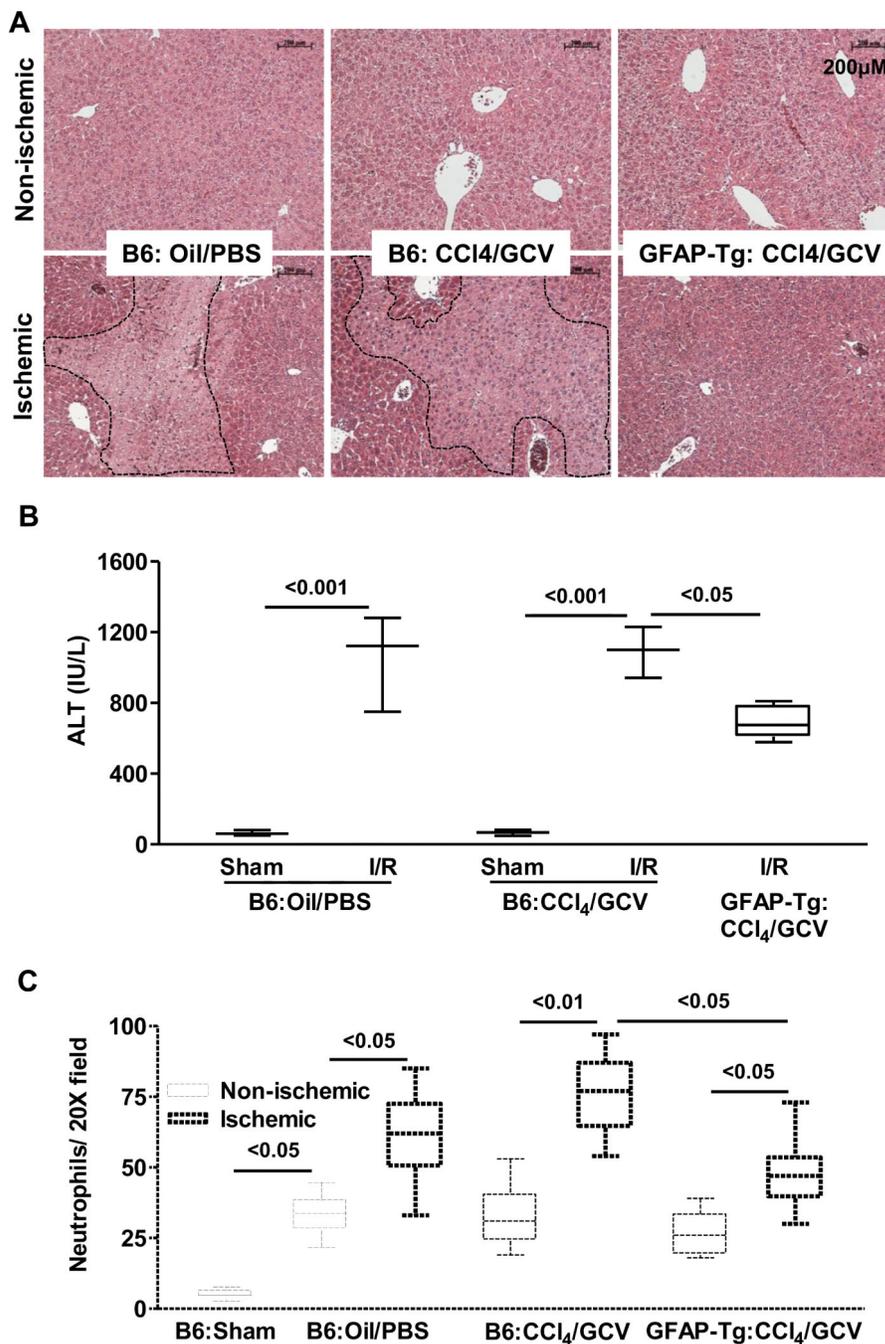


Fig. 2. Hepatic I/R injury in HSC-depleted mice
 WT-B6 and GFAP-Tg mice (n=4-6) were treated with oil or CCl₄ (3 injections) followed by its discontinuation and PBS or GCV treatment of 10 days. They were then subjected to hepatic I/R. (A) Histopathology, (B) ALT and (C) neutrophil infiltration are shown.

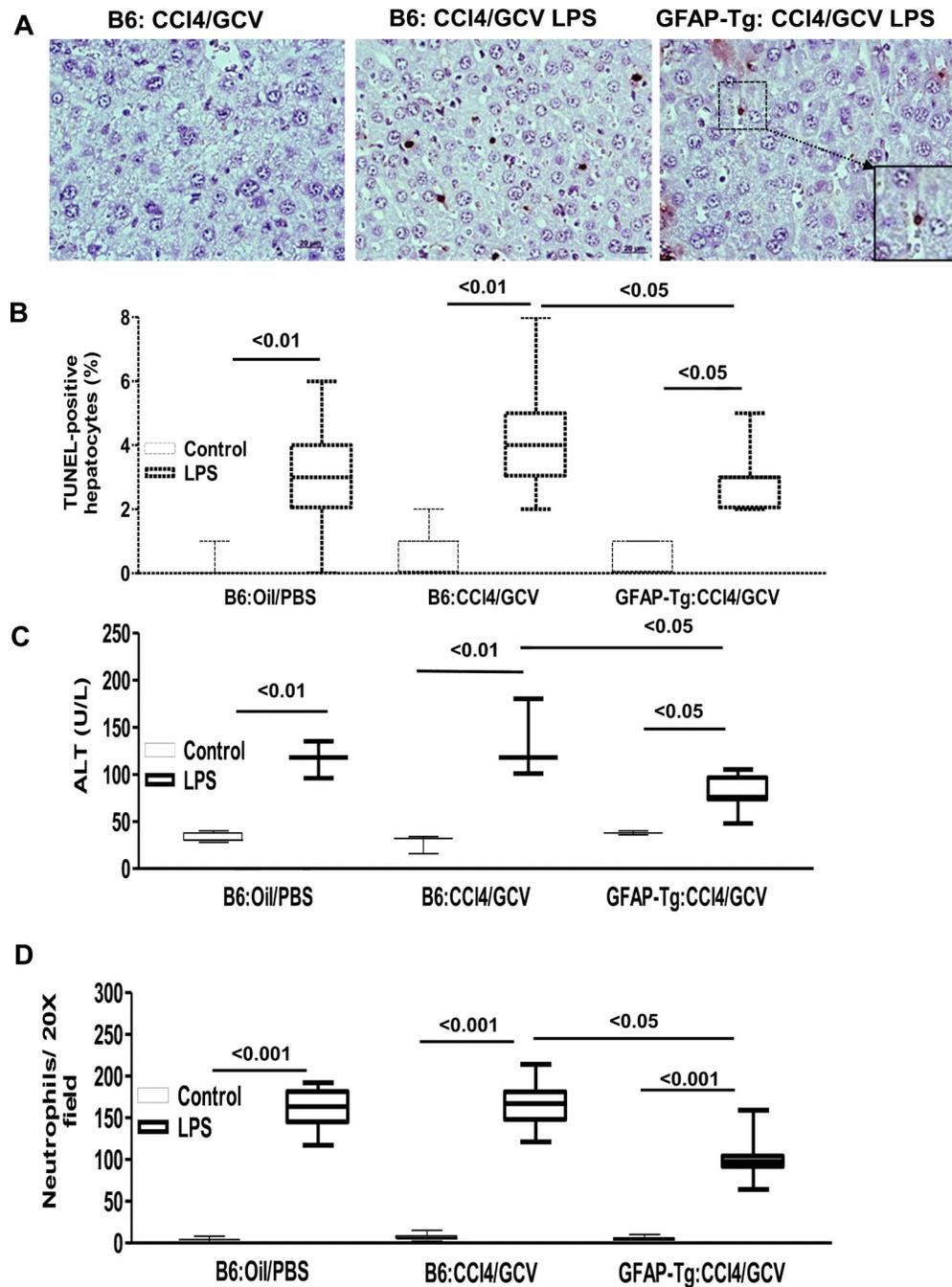


Fig. 3. Hepatic endotoxemic injury in HSC-depleted mice

WT-B6 and GFAP-Tg mice (n=4-6) received CCI4 (3 injections) followed by its discontinuation and GCV treatment of 10 days, then subjected to LPS treatment. (A) Histopathology and (B) quantification of TUNEL- positive hepatocytes; (C) serum ALT; and (D) neutrophil infiltration. Compared to CCI4/GCV then LPS-treated B6 mice very few apoptotic hepatocytes were observed in the livers of CCI4/GCV then LPS-treated GFAP-Tg mice; inset shows high magnification of the marked area to show apoptotic HSC.

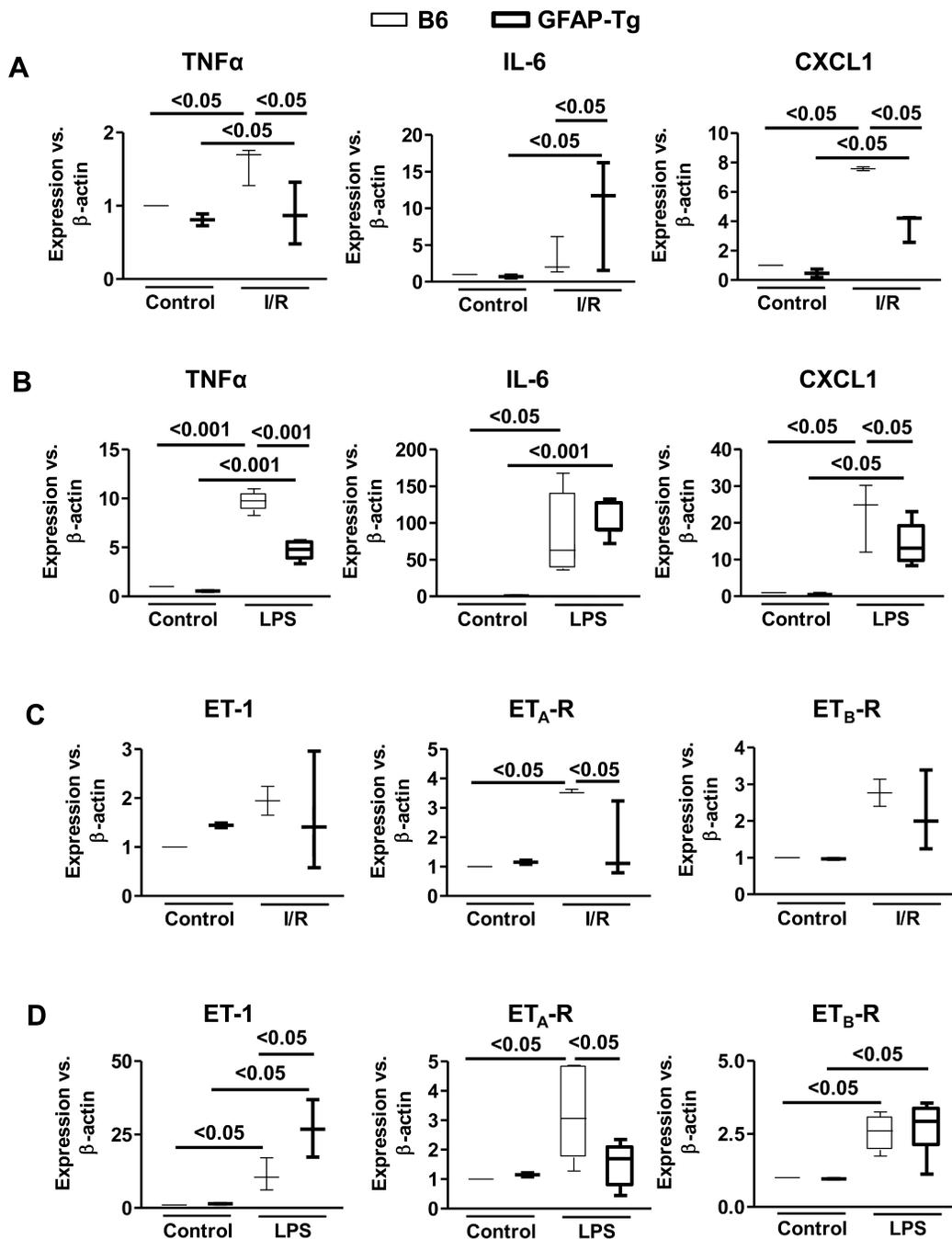


Fig. 4. Hepatic mRNA expression of molecules of I/R- or endotoxin-induced injury in HSC-depleted mice

WT-B6 and GFAP-Tg mice ($n=4-6$) received CCl₄ (3 injections) followed by its discontinuation and GCV treatment of 10 days, then subjected to I/R or LPS treatment. The mRNA expression of (A, B) TNF- α , IL-6 and CXCL1 or (C, D) ET-1, ET_A-R and ET_B-R were quantified in the nonischemic and ischemic lobes (A, C) and livers of control and LPS-treated mice (B, D).

Table 1

Primers used in qPCR reactions

GFAP	5'-ACGCATCACCATTCTGTAC-3'(F) 5'-TTGCCTTCTGACACGGATT-3'(R)
IL-6	5'-CCGGAGAGGAGACTTCACAG-3'(F) 5'-TCCACGATTTCCAGAGAAC-3'(R)
TNF α	5'-CCCAGGTATATGGGCTCATACC-3'(F) 5'-GCCGATTGCTATCTCATACCAGG-3'(R)
CXCL1	5'-CTGCACCCAAACCGAAGTC-3'(F) 5'-AGCTTCAGGGTCAAGGAAG-3'(R)
ET-1	5'-GTGTCTACTTCTGCCACCTGGACAT-3'(F) 5'-GGGCTCGCACTATATAAGGGATGAC-3'(R)
ET _A R	5'-GATGGATAAGAACCGGTGTGAAC-3'(F) 5'-GAGCTATTGGGTTTATGCAAGAATTC-3'(R)
ET _B R	5'-ACCAGAGCAATCCACACAGG-3'(F) 5'-AGAGCGATTGGATTGATGCAG-3'(R)
GAPDH	5'-TGTTGAAGTCACAGGAGACAACCT-3'(F) 5'-AACCTGCCAAGTATGATGACATCA-3'(R)