Published in final edited form as:

J Cell Physiol. 2016 January ; 231(1): 94–105. doi:10.1002/jcp.25055.

Endotoxin-stimulated Rat Hepatic Stellate Cells Induce Autophagy in Hepatocytes as a Survival Mechanism

Anil Dangi^{1,2,3}, Chao Huang¹, Ashish Tandon^{1,2}, Donna Stolz⁴, Tong Wu⁵, and Chandrashekhar R. Gandhi^{1,2,3,6,*}

¹Thomas E. Starzl Transplantation Institute, Department of Surgery, University of Pittsburgh, Pittsburgh, Pennsylvania

²Department of Surgery, University of Cincinnati, and Cincinnati VA Medical Center, Cincinnati, Ohio

³Cincinnati VA Medical Center, Cincinnati, Ohio

⁴Department of Cell Biology, University of Pittsburgh, Pittsburgh, Pennsylvania

⁵Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, Louisiana

⁶Department of Pediatrics, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio

Abstract

Bacterial lipopolysaccharide (LPS)-stimulated hepatic stellate cells (HSCs) produce many cytokines including IFN β , TNF α , and IL6, strongly inhibit DNA synthesis, but induce apoptosis of a small number of hepatocytes. In vivo administration of LPS (up to 10 mg/mL) causes modest inflammation and weight loss in rats but not mortality. We determined whether LPS-stimulated HSCs instigate mechanisms of hepatocyte survival. Rats received 10 mg/kg LPS (i.p.) and determinations were made at 6 h. In vitro, HSCs were treated with 100 ng/mL LPS till 24 h. The medium was transferred to hepatocytes, and determinations were made at 0–12 h. Controls were HSC-conditioned medium or medium-containing LPS. LPS treatment of rats caused autophagy in hepatocytes, a physiological process for clearance of undesirable material including injured or damaged organelles. This was accompanied by activation of c-Jun NH2 terminal kinase (JNK) and apoptosis of ~4–5% of hepatocytes. In vitro, LPS-conditioned HSC medium (LPS/HSC) induced autophagy in hepatocytes but apoptosis of only ~10% of hepatocytes. While LPS/HSC stimulated activation of JNK (associated with cell death), it also activated NF κ B and ERK1/2 (associated with cell survival). LPS-stimulated HSCs produced IFN β , and LPS/HSC-induced autophagy in hepatocytes and their apoptosis were significantly inhibited by anti-IFN β antibody. Blockade of autophagy, on the other hand, strongly augmented hepatocyte apoptosis. While LPS-stimulated HSCs cause apoptosis of a subpopulation of hepatocytes by producing IFN β , they also induce cell

*Correspondence to: Chandrashekhar R. Gandhi, Department of Surgery ML0558, University of Cincinnati, 231 Albert Sabin Way, Cincinnati, OH, 45267-0558. gandhicr@ucmail.uc.edu.

Conflict of Interest: None.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site

survival mechanisms, which may be of critical importance in resistance to liver injury during endotoxemia.

The liver is constantly exposed to noxious substances from the gastrointestinal organs, including gram-negative bacterial endotoxin (lipopolysaccharide; LPS). LPS levels increase during hepatic injury and inflammation, and it may act directly on hepatocytes or via soluble mediators released by nonparenchymal cells such as Kupffer cells and hepatic stellate cells (HSCs). Previously, we found that LPS administration to rats caused mild liver injury and weight loss but all animals survived the endotoxin challenge (Gandhi et al., 2001). These observations suggested that both death-inducing and survival signals are stimulated by LPS in hepatocytes, with predominance of the latter.

The perisinusoidal HSCs (about 10% of the liver cell population) are a major storage site of body's retinoids in physiology; during liver injury, the quiescent HSCs transdifferentiate into activated α -smooth muscle actin (α -sma)-expressing proliferating myofibroblasts and become a major cell type of hepatic fibrosis (Gandhi, 2010; Puche et al., 2013; Hasegawa et al., 2015). We found that LPS-stimulated quiescent and activated murine HSCs produce nitric oxide (NO), many cytokines including interleukin-6 (IL6), tumor necrosis factor- α (TNF α), IL10, and interferon- β (IFN β) and several chemokines (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005, 2006; Dangi et al., 2012; Harvey et al., 2013). Thus, increased NO, IL6, and TNF α in human and experimental endotoxemia (Decker, 1998; Fukui, 2005; Bilzer et al., 2006) suggest contribution by HSCs to their elevation, and the role of these cells in regulating hepatocyte survival and function. Indeed, strong inhibition of DNA synthesis but apoptosis of only a small percentage of hepatocytes by LPS-stimulated HSCs (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005) indicate instigation of survival processes in the majority of hepatocytes.

Autophagy is a physiological process that prevents cell injury by clearing undesirable material including abnormal or misfolded proteins and injured/damaged organelles (Klionsky et al., 2008; Mizushima et al., 2010; Czaja et al., 2013). However, cells undergo apoptosis upon overproduction of autophagic vesicles that interferes with normal membrane trafficking, or upon blockade of autophagocytic degradation of abnormal proteins and damaged organelles (Klionsky et al., 2008; Czaja et al., 2013).

We hypothesized that LPS-stimulated HSCs (LPS/HSC), in addition to pro-apoptotic factors, may also produce mediators that increase autophagy in hepatocytes as a survival mechanism during increased endotoxin levels. We found that LPS/HSC produced IFN β and increased the number of hepatocytes with active autophagy. Endotoxin administration to rats also increased IFN β expression and induced autophagy, but caused apoptosis of a small subpopulation of hepatocytes. Thus predominance of pro-survival over pro-apoptotic mechanisms by LPS-stimulated HSCs in hepatocytes may have important implications in restricting liver injury during endotoxemia.

Materials and Methods

Reagents

The following were purchased from indicated sources: LPS (*E. coli* lipopolysaccharide serotype 0111:B4), RIPA buffer, anti- β -actin antibody (Ab), baflomycin, and chloroquine (Sigma-Aldrich, St. Louis, MO); anti-desmin Ab (Abcam, Cambridge, MA), recombinant IFN β , and neutralizing Ab (InterferonSource, Piscataway, NJ); anti-caspase-3, -LC3, P-eIF2 α , -P-PERK, -CHOP, -P-ERK1/2, -JNK, -P-JNK, and (S473)-P-AKT Abs (Cell Signaling, Beverly, MA), and JNK inhibitor (SP600125) (Calbiochem, La Jolla, CA); anti-ERK1/2 and anti-NF- κ B p65 Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Culture and treatments of HSCs and hepatocytes

Animal protocols were approved by Institutional Animal Care and Use Committees according to NIH guidelines. HSCs were isolated from male Sprague-Dawley rats (450–500 g), purified using Nycodenz gradient and cultured as described previously (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005, 2006). The medium was renewed after overnight culture, then on alternate days, and the cells were used on day 7 of culture.

Hepatocytes were prepared by collagenase digestion of the rat liver, purified on Percoll gradient, and cultured as described previously (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005). The medium was renewed after 3 h attachment period and the cells were used after overnight incubation.

HSCs were incubated in DMEM containing 5% FBS, without or with 100 ng/mL LPS for up to 24 h (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005). Sterile-filtered HSC-conditioned medium (without or with LPS) was transferred to hepatocytes. Control hepatocytes were placed in medium preincubated without or with LPS in the absence of HSCs.

LPS treatment of rats

LPS (10 mg/kg) was administered intraperitoneally to male Sprague-Dawley rats (200–250 g) to induce endotoxemia (Geller et al., 1994). At 6 h, portions of right hepatic lobe were fixed in buffered formalin, 2% paraformaldehyde, 2.5% glutaraldehyde, or snap-frozen in liquid nitrogen. Formalin-fixed liver tissue was embedded in paraffin, and sections were stained with hematoxylin/eosin.

Immunocytochemistry

HSCs were treated with 1 μ L/mL brefeldin A (Biolegend) $\pm$ 100 ng/mL LPS for 6 h, washed with ice-cold phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde. After permeabilization and blocking with PBS containing 1% bovine serum albumin (BSA), 10% goat serum, 0.3 M glycine, and 0.1% Tween-20, the cells were treated with anti-IFN β and anti- α -sma (DAKO) Abs for 24 h at 4°C, washed, and incubated in secondary goat anti-rabbit-AlexaFluor-594 and goat anti-mouse-AlexaFluor-488 (Invitrogen, Grand Island, NY) for 1 h at room temperature. The cells were then washed with PBS, treated with 4',6-diamidino-2-phenylindole (DAPI) and mounted.

For LC3 staining, hepatocytes were washed with PBS, fixed in 2% paraformaldehyde in PBS, permeabilized with 0.1% triton X-100, and washed with PBS followed by 0.5% BSA in PBS (PBB). After blocking in 2% BSA, the cells were washed with PBB, incubated in PBB containing anti-LC3 Ab (NB100-2220; NOVUS Biologicals, Littleton, CO) (1:500 v/v), washed with PBB, and incubated with goat anti-rabbit Alexa Fluor-488 (1:1000 v/v). After washing with PBB and nuclear staining with DAPI, the cells were washed with PBS, and coverslipped with Gelvatol.

Western blot analysis

Cell lysates were prepared in RIPA buffer containing 50 mM glycerophosphate, 20 mM NaF, 1 mM Na₃VO₄, 2 mM phenylmethylsulfonylfluoride, 10 µg/mL leupeptin and 10 µg/mL aprotinin. Proteins were separated by SDS-PAGE and transferred to PVDF membrane. After immunoblotting with primary Abs, washing and incubation with appropriate secondary Abs, protein bands were detected using ECL reagent (GE Healthcare, Buckinghamshire, UK). β -actin expression ensures equal protein loading.

For determination of nuclear translocation of NF- κ B by Western analysis, nuclear, and cytoplasmic extracts were prepared using extraction reagents from Pierce-Endogen (Rockford, IL). Nuclear (5 µg) and cytoplasmic (20 µg) proteins were separated by 10% SDS-PAGE. Western blotting was performed using anti-NF- κ B p65 Ab (Santa Cruz; 1:1000) and secondary Ab (1: 20,000).

mRNA analysis

RNA was extracted and cDNA was synthesized for semi-quantitative RT-PCR essentially as described previously (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005; 2006) using the following primers. IFN- β : 5'-CGTTCCTGCTGTGCTTCTC-3' (F) and 5'-TGTAACCTCTTCTCCATCTGTGAC-3' (R); GAPDH: 5'-ATGCCATCACTGCCACTC-3' (F) and 5'-GGGTAGGAACACGGAAGG-3' (R). Initial denaturation was done at 94°C for 5 min, followed by 30 cycles at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. Quantitative-RT-PCR (qRT-PCR) analysis was performed as described previously (Dangi et al., 2012; Harvey et al., 2013) using primers for IFN β : 5'-GGTGACATCCACGACTACTTTAG-3' (F) and 5'-CCAGGCATAGCTGTTGTACTT-3' (R); GAPDH: 5'-CCATGGAGAAGGCTGGGG-3' (F) and 5'-CTGCCCCACGGCCATCAC-3' (R).

Determination of caspase-3 activity and apoptosis

Caspase-fluorescent assay kit (BD Biosciences-Clontech) was used to measure Caspase-3 or DEVD-cleaving activity, an early marker of cells undergoing apoptosis. The activity was measured as caspase3-mediated release of fluorescent 7-amino-4-methylcoumarin from the substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC). Apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay (Thirunavukkarasu et al., 2005) as per the manufacturer's instructions (Roche Pharmaceuticals, Nutley, NJ). TUNEL-positive nuclei were detected by fluorescent microscope. Cellular release of lactate dehydrogenase (LDH), a marker of membrane damage and necrosis, was determined using a kit from Stanbio Laboratory (Boerne, TX).

Determination of DNA synthesis

DNA synthesis was measured as described previously (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005). Cells were incubated in DMEM/0.1% BSA containing 1 $\mu\text{Ci/mL}$ [methyl- ^3H] thymidine (3.18TBq/mmol) (Amersham-Pharmacia, Piscataway, NJ) for 4 h at 37°C, then washed with ice-cold HBSS/BSA, treated with ice-cold 10% TCA for 10 min, and washed once with TCA followed by 95% ethanol. Cells were digested with 5% SDS for determination of radioactivity.

Flow cytometric cell cycle analysis

The medium containing detached cells was aspirated, and the attached cells were harvested using trypsin. The two cell fractions were pooled, washed twice with PBS by centrifugation at 450g (10 min, 4°C), fixed in ice-cold 70% ethanol for 3 h, and washed with Ca^{2+} - and Mg^{2+} - free HBSS containing 1% BSA. The cells were suspended in 0.5 mL of propidium iodide solution (50 $\mu\text{g/mL}$ propidium iodide, 1 mg/mL sodium citrate, 100 $\mu\text{g/mL}$ RNase I, and 0.1% triton X-100). After 30 min of incubation at 37°C, the cells were analyzed by flow cytometry in a fluorescence-activated cell sorter (Epics XL.MCL, Beckman Coulter using EXPO32 software). The subG0 phase, G0/G1, S, and G2/M phases of the cell cycle were analyzed by diploid staining profiles and ModFit software program. Forward and side light scatter characteristics were used to exclude cell debris (size and granularity) from the analysis. The percentage of cell aggregates (doublets) and other background were also eliminated using peak versus integral gating of cells.

Transmission Electron Microscopy (TEM)

Cultured cells or 1 mm³ cubes of the liver tissue were fixed overnight in 2.5% glutaraldehyde in PBS at 4°C, washed, and post-fixed in aqueous 1% OsO_4 , 1% $\text{K}_3\text{Fe}(\text{CN})_6$. Following 3 PBS washes, the samples were dehydrated through a graded series of 30–100% ethanol, 100% propylene oxide then infiltrated in 1:1 mixture of propylene oxide:Polybed 812 epoxy resin (Polysciences, Warrington, PA) overnight at room temperature. After several changes of 100% resin over 24 h, the samples were embedded in molds, cured at 37°C overnight, followed by additional hardening at 65°C for 2 days. Ultrathin (70 nm) sections were collected on 200 mesh copper grids, stained with 2% uranyl acetate in 50% methanol (10 min), followed by 1% lead citrate (7 min). Sections were imaged using a JEOL JEM 1011 transmission electron microscope (Peabody, MA) at 80 kV fitted with a side mount AMT 2 k digital camera (Advanced Microscopy Techniques, Danvers, MA).

Confocal microscopy

Paraformaldehyde-fixed tissue was placed in 30% sucrose for 24 h, then in 2-methylbutane in a metal dish containing liquid nitrogen until frozen. Cryostat sections (5 micron) of the fixed tissue were washed with PBS, then with 0.5% BSA in PBS. Sections were blocked with 2% BSA for 30 min, incubated for 1 h at room temperature with anti-desmin Ab (1:100 in 0.5% BSA), washed with 2% BSA and incubated for 1 h with goat anti-mouse Alexa Fluor[®] 488 (1:500 in BSA), in combination with 1:250 dilution of F-actin dye Rhodamine Phalloidin (Invitrogen). Nuclei were stained with Hoeschst dye (1 mg/100 mL water) for 30 sec. Sections were treated with TUNEL stain, washed with PBS then coverslipped with

Gelvatol. Fluorescent images were captured with an Olympus Fluoview 1000 confocal microscope.

Statistical analysis

In vitro experiments were performed at least 3 times, each in duplicate or triplicate using cells from different animals. In vivo experiments were performed using 4–6 animals/group. Results are expressed as means $\pm$ SD. Statistical significance between groups was determined by Student's *t*-test using Prism 5 Software package (GraphPad Software, GraphPad, San Diego, CA) or by Kruskal-Wallis One-Way ANOVA on Ranks followed by Dunn's method post-hoc test. A "*P*" value of <0.05 was considered statistically significant.

RESULTS

LPS-stimulated HSCs induce autophagy in hepatocytes

TEM of hepatocytes treated with LPS/HSC (Fig. 1d–h), but not those treated with LPS or Control/HSC (Fig. 1a–c), showed several autophagosomes containing cytosolic material including glycogen (Fig. 1e–g), mitochondria (g and h) and endoplasmic reticulum (h). Quantification showed a significant increase in the number of autophagic vesicles in LPS/HSC-challenged hepatocytes.

During autophagy microtubule-associated protein 1 light chain-3 (LC3 or Atg8) is converted to phosphatidylethanolamine-conjugated membrane-bound LC3-II. Localization/accumulation of LC3-II in autophagosomes is required for the formation and elongation of their double-membrane (Klionsky et al., 2008; Mizushima et al., 2010; Czaja et al., 2013). The number of hepatocytes containing LC3 puncta as well as the number of LC3 puncta/hepatocyte increased upon their incubation in LPS/HSC medium (Fig. 2A). LC3-II expression increased in LPS/HSC-stimulated hepatocytes within 1 h and remained similarly elevated at 6 h (Fig. 2B and Supplemental Figure S1A). In contrast to the previous findings that increase in LC3-II during autophagy is associated with a concomitant decrease in p62 (Klionsky et al., 2008; Mizushima et al., 2010; Czaja et al., 2013), no significant change was observed in p62 in LPS/HSC-stimulated hepatocytes (Fig. 2B). Such effect on LC3-II and p62 has recently been reported (Chen et al., 2014), and thus the steady state level of p62 during LPS/HSC treatment of hepatocytes (Fig. 2B) reflects the autophagic status (Wang et al., 2006; Komatsu et al., 2007). We also incubated hepatocytes with HSC-medium conditioned by LPS for 1–24 h; within 1 h during conditioning, LPS induced release of mediators that caused LC3-II formation in hepatocytes (Supplemental Figure S1B).

Next we determined autophagy flux using baflomycin (inhibits autolysosome formation and lysosomal degradation) or lysosomal inhibitors chloroquine (inhibits autolysosome formation) (Klionsky et al., 2008; Mizushima et al., 2010; Czaja et al., 2013). Preliminary experiments demonstrated loss of hepatocyte viability with 10 nM baflomycin and 50 μ M chloroquine at 6 h (Supplemental Figure S2A). Therefore, we used 1 nM baflomycin and 10 μ M chloroquine, and found further increase in LC3-II expression in LPS/HSC-treated hepatocytes in presence of baflomycin (Fig. 2C) and chloroquine (Supplemental Figure S2B); both inhibitors also increased p62 expression. Since inhibition of autophagy increases

accumulation of p62, these data suggest a role of autophagy in mediating its clearance (Hara et al., 2006; Komatsu et al., 2006; Szeto et al., 2006; Kaniuk et al., 2007). Presence of baflomycin increased LC3-II similarly in control hepatocytes and those incubated with LPS or in HSC-conditioned medium (Supplemental Figure S2C) indicating ongoing autophagy at a low level physiologically. 3-Methyladenine (a phosphoinositide-3-kinase [PI3K] inhibitor that prevents pre-autophagosomal structure formation) prevented LPS/HSC-induced LC3-II formation and increased p62 expression (Fig. 2C). Furthermore, 3-MA increased apoptosis of hepatocytes under control, LPS-stimulated and HSC-stimulated conditions, and caused robust increase in apoptosis of LPS/HSC-treated hepatocytes (Fig. 3).

c-Jun NH2-terminal kinase (JNK) mediates LPS/ HSC-induced apoptosis and autophagy in hepatocytes

Cellular stress is associated with JNK activation, and evidence indicates a role for JNK in induction of autophagy and apoptosis (Gunawan et al., 2006; Ni et al., 2008; Lorin et al., 2010; Kim et al., 2013; Jia et al., 2014). Both JNK1 and JNK2 activation occurred in LPS/ HSC-treated hepatocytes (Fig. 4A). Time-course of this effect demonstrated increased JNK1 and JNK2 phosphorylation within 1 h of LPS/HSC-stimulation of hepatocytes, which declined subsequently from 3 h (Supplemental Figure S3A). Since activation of AKT (protein kinase B) has been reported to show reciprocal relationship with JNK activation, and is considered a survival signaling mechanism (Davis, 2000; Aikin et al., 2004; Hers et al., 2011; Martinovic et al., 2012), we assessed AKT phosphorylation under the experimental conditions. As shown in Figure 4A, AKT phosphorylation reduced in hepatocytes upon incubation with LPS/HSC stimulation.

Because the strong inhibition of DNA synthesis in hepatocytes by LPS/HSC (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005) indicated possible cell cycle arrest, effect of JNK inhibitor (SP600125) on the cell cycle of LPS/HSC-challenged hepatocytes was determined via flow cytometry. LPS itself increased the subG0 phase indicative of growth arrest (Diez-Fernandez et al., 1998), and this was prevented by SP600125 (Supplemental Figure S4, upper panel). HSC-conditioned medium also increased subG0 peak but did not affect significantly the S phase. LPS/HSC caused further increase in subG0 peak and reduction in the S phase; this effect was reversed by SP600125 (Supplemental Figure S4; lower panel).

LPS/HSC induced caspase-3 activation (Fig. 4B) and caused apoptosis of a subpopulation of hepatocytes (Fig. 4C), which were both reversed partially by SP600125. No necrosis of hepatocytes was observed histologically and by cellular release of lactate dehydrogenase (a marker of membrane damage and necrosis) (results not shown). LPS/HSC-induced JNK activation was completely prevented by SP600125 (Fig. 4B). Furthermore, LPS/HSC-induced autophagy in hepatocytes was also inhibited by SP600125 (Fig. 4D) suggesting that JNK may be a common signaling pathway for induction of autophagy and apoptosis of hepatocytes.

LPS-stimulated HSCs activate ERK1/2 and NFkB in hepatocytes

Because apoptosis of only up to 10% hepatocytes occurred due to LPS/HSC, we examined whether extracellular signal-regulated kinase (ERK1/2), an important signaling pathway of cell survival (Cobb et al., 1994; Chen et al., 2001; Pearson et al., 2001), is activated in LPS/HSC-stimulated hepatocytes. LPS/HSC stimulated ERK1/2 activation, which was still persistent at 12 h (Supplemental Figure S3B), unlike the decline in JNK activation (Supplemental Figure S3A). HSC as well as LPS/HSC also stimulated NFkB nuclear translocation (another cell survival signaling pathway) (Amsterdam et al., 2002; McMillian et al., 2005; Strozyk and Kulms, 2005), but whereas HSC-induced NFkB activation returned to basal level by 4 h, that by LPS/HSC remained high (Supplemental Figure S3C).

IFN β mediates LPS/HSC-induced apoptosis of hepatocytes

To determine the mechanism of LPS/HSC-induced autophagy in hepatocytes and their apoptosis, we examined HSC-derived cytokines that can potentially cause these effects. Recently, we found transient but robust increase in IFN β mRNA expression in LPS-stimulated HSCs (Harvey et al., 2013). Because type I interferons are implicated in liver injury (Geller et al., 1993; Tsung et al., 2006; Dhupar et al., 2011) and autophagy (Schmeisser et al., 2013, 2014), we determined IFN β expression in LPS-stimulated HSCs. A strong increase in IFN β mRNA expression occurred by 1–3 h in LPS-stimulated HSCs that decreased markedly at 6 h and returned to basal level at 12 h (Fig. 5A). This effect was reflected in accumulation of IFN β protein in LPS-stimulated HSCs (Fig. 5A). Exogenously added IFN β increased LC3-II formation in hepatocytes (Fig. 5B) and LPS/HSC-induced LC3-II formation was inhibited by anti-IFN β Ab (Fig. 5C). IFN β increased JNK activation in hepatocytes (Fig. 5D); interestingly, anti-IFN β Ab inhibited LPS/HSC-induced activation of JNK1, but strongly increased JNK2 activation beyond that by LPS/HSC (Fig. 5D).

IFN β inhibited DNA synthesis in hepatocytes (Fig. 6A), induced caspase-3 activation (Fig. 6B), and caused apoptosis of 10–15% of the cells (Fig. 6C), and blockade of IFN β inhibited LPS/HSC-induced apoptosis of hepatocytes (Fig. 6D). These data suggest that IFN β is a major mediator of LPS/HSC-induced apoptosis of hepatocytes.

Hepatocytes in LPS-treated rats exhibit apoptosis, ER stress, and autophagy

Next, we determined in vivo relevance of the in vitro findings. LPS treatment caused significant weight loss (Supplemental Figure S5A) and increase in ALT (Supplemental Figure S5B), sinusoidal dilation, and mild inflammatory infiltration (Fig. 7A-a [PBS treatment] vs. 7A-b [LPS treatment]). Confocal microscopy of LPS-treated liver revealed apoptosis of hepatocytes ($4 \pm 1/100$ hepatocytes), and significant number of the apoptotic cells were in close proximity to HSCs (Fig. 7A-c [PBS-treated] vs. 7A-d [LPS-treated]). TEM of LPS-treated livers showed dilation of ER with loss of ribosomes and formation of autophagosomes in hepatocytes (Figs. 7B-b and 7B-d). The autophagosomes contained cytosolic material, mitochondria as well as endoplasmic reticulum (ER) (Figs. 7B-b, 7B-e, and 7B-f). There was also marked formation of cytosolic vacuoles and ER dilation indicating ER stress (Figs. 7B-b, 7B-d, and 7B-f). These TEM findings were consistent with increased LC3-II formation in hepatocytes isolated from the LPS-treated rat liver (Fig. 7C). ER stress was also confirmed by increased phosphorylation of the double stranded RNA-

activated protein kinase-like ER kinase (PERK) and α -subunit of eukaryotic translation initiation factor 2 (eIF2 α) and c/EBP homologous protein-10 (CHOP/GADD153) expression (ER stress markers) (Ma and Hendershot, 2002; Gotoh and Mori, 2006) (Fig. 7C). Furthermore, IFN β mRNA expression increased within 1 h, and declined to undetectable level at 3–6h in LPS-treated liver (Fig. 7D).

To confirm contribution of HSCs to autophagy induced by LPS in the liver, we used our recently developed HSC-depleted mouse model (Stewart et al., 2014). LPS-induced injury was found to be markedly reduced as compared to control mice (Stewart et al., 2014). Challenging HSC-sufficient mice with LPS increased hepatic LC3-II formation, and magnitude of this effect reduced in HSC-depleted mice (Fig. 8).

Discussion

The liver has an extraordinary capacity to degrade LPS to which it is exposed almost continuously. Even administration of high dose of LPS (e.g., 10 mg/kg) exerts modest hepatic effects as indicated by moderate inflammation and low level of apoptosis (Fig. 7). All of the rats administered LPS survive this insult. The weight loss observed in LPS-treated rats can be attributed to dehydration and reduced food intake (Farges et al., 1996; Sah et al., 2011). These observations suggest that while death-inducing stimuli operate within the liver, contemporaneous activation of the survival signals overcome LPS-induced liver injury. The present work provides evidence that LPS-stimulated HSCs produce soluble mediators that are able to induce such signals in hepatocytes, with predominance of survival pathways over the apoptotic pathways. However, a possibility cannot be excluded that heterogeneity in hepatocytes may reflect the susceptibility of a minor population to undergo apoptosis.

Previously, we reported that LPS-stimulated HSCs strongly inhibit DNA synthesis in hepatocytes and induce apoptosis of a small percentage of the cells in vitro, and these effects could not be overcome by the addition together of the antibodies to TNF α , IL1 β , and TGF β + an inhibitor of NO synthase (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005). These data suggested that HSCs release other factors that mediate hepatocyte apoptosis. Here we found that IFN β produced by LPS-stimulated HSCs causes apoptosis of hepatocytes. LPS/ HSC as well as IFN β activated JNK, an established signaling pathway of hepatocyte apoptosis (Ding and Yin, 2004; Gunawan et al., 2006; Ni et al., 2008; Speicher et al., 2012). Interestingly, anti-IFN β Ab prevented JNK1 activation but strongly increased JNK2 activation beyond that by LPS/HSC. It is shown that signaling via JNK2 stimulates protective mechanisms whereas JNK1 signaling is cytotoxic to hepatocytes (Qiao et al., 2003; Raciti et al., 2012). Thus increased activation both of JNK1 and JNK2 by LPS/HSC in hepatocytes indicates simultaneous stimulation of apoptotic and survival signals, and inhibition of IFN β channels this to a dominant survival mechanism by increasing JNK2 activation. However, it should also be noted that JNK1 and JNK 2 have overlapping functions ranging from cell activation, differentiation, proliferation, and tumorigenesis to apoptosis in various cell types (Sabapathy et al., 2004). Furthermore, JNK2 has been reported to negatively regulate proliferation and JNK1 induced a positive effect in primary murine embryonic fibroblasts (MEFs) and immortalized 3T3 fibroblast cell lines (Tournier et al., 2000; Sabapathy et al., 2004). These observations indicate that cell-specific differences

in the effects of JNK1 and JNK2; precise determination of how JNK1 and JNK2 regulate HSC/hepatocyte interactions during endotoxemia remains to be determined.

Another mechanism of the survival of the majority of hepatocytes challenged by LPS/HSC could be persistently activated ERK-MAPK and NFkB. ERK1/2 are members of the mitogen-activated protein kinase (MAPK) family whose activation is coupled to cell growth, proliferation, and survival (Cobb et al., 1994; Chen et al., 2001; Pearson et al., 2001). NFkB signaling is also known to be an important pathway of cell survival (Amsterdam et al., 2002; McMillian et al., 2005; Strozyk and Kulms, 2005), and its activation has been linked to transformation, proliferation, suppression of apoptosis, invasion, angiogenesis, and metastasis (Aggarwal, 2004). Mediators such as TNF α and IL10 have been shown to stimulate ERK1/2 and NFkB signaling (Roberts et al., 2000; Santiago-Lomelí et al., 2005; Schwabe and Brenner, 2006). IL10 protects hepatocytes during endotoxin-induced liver injury (Arai et al., 1995), I/R injury (Dinant et al., 2007; Li et al., 2009), and IL6, a priming agent for hepatocyte proliferation during liver regeneration (Fausto et al., 1995; Michalopoulos and DeFrances, 1997), also protects against I/R injury (Camargo et al., 1997). Since LPS stimulates synthesis IL6 and IL10 in HSCs (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005, 2006; Dangi et al., 2012; Harvey et al., 2013), these cytokines may provide a cell survival mechanism possibly via ERK1/2 and NFkB activation in LPS/HSC-challenged hepatocytes and in endotoxemia.

Autophagy is a physiological process of clearance of undesired material, and this protects the cells from accumulation of injurious material. But persistent or excessive autophagy leads to cell death. Thus LPS/HSC-induced autophagy in hepatocytes in vitro as well as LPS-induced autophagy in vivo can be interpreted to impart protection from damage. Indeed, previous work suggested autophagy as a protective mechanism during acetaminophen-induced toxicity (Ni et al., 2012). Our results showing robust apoptosis of hepatocytes incubated in LPS/HSC medium in presence of 3-methyladenine emphasize that autophagy induced by LPS/HSC is primarily protective. Our results further indicate that this mechanism may be provided through HSC-derived IFN β , which induced apoptosis of only a subpopulation of hepatocytes; the effect is reversed by anti-IFN β Ab. On the other hand, anti-IFN β Ab partially reversed LPS/HSC-induced LC3-II formation. Because LPS-stimulated HSCs produce many cytokines (Harvey et al., 2013), LPS/HSC-induced autophagy in hepatocytes may be a cumulative effect of several factors including IL6 (Harris, 2011). Since excessive autophagy can lead to cell death, limited apoptosis of hepatocytes by LPS in vivo and by LPS/HSC in vitro suggest that autophagy was self-limiting. Indeed, both bafilomycin and chloroquine strongly increased LC3-II accumulation, and blockage of autophagy with 3-methyladenine led to robust apoptosis. It is also plausible that mediator such as IL10, which is shown to inhibit autophagy (Harris, 2011), produced by LPS-stimulated HSCs (Dangi et al., 2012; Harvey et al., 2013) is responsible in preventing excessive autophagy. Significantly reduced LPS-induced liver injury (Stewart et al., 2014) and autophagy in HSC-depleted mice (Fig. 8) than in control mice further support the role of HSCs in these effects.

The results of this study may have important clinical relevance to LPS-induced liver injury. Since the liver is exposed to low level of LPS on a continuous basis, vulnerability of

hepatocytes to death-inducing mediators will be enhanced upon increase in portal LPS due to increased gut permeability (e.g., in alcohol-induced injury) or in disorders in which liver's functions are compromised. Release of soluble mediators by LPS-stimulated HSCs (Uemura and Gandhi, 2001; Thirunavukkarasu et al., 2005, 2006; Dangi et al., 2012; Harvey et al., 2013), which are located in close proximity of hepatocytes, will be critical to prevent excessive liver injury in such conditions. However, a possibility that HSCs may render hepatocytes vulnerable to death-inducing stimuli by promoting ER stress/autophagy should be considered. Indeed, mice pretreated with LPS undergo extensive liver injury upon subsequent treatment with otherwise innocuous dose of acetaminophen (Maddox et al., 2010).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Contract grant sponsor: VA Merit Review Award;

Contract grant number: 1H01BX001174-01.

Contract grant sponsor: NIH;

Contract grant numbers: PO1AIO81678, R21AA020846.

Abbreviations

Ab	antibody
HSC	hepatic stellate cell
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

Literature Cited

- Aggarwal BB. Nuclear Factor-kB: The enemy within. *Cancer Cell*. 2004; 6:203–208. [PubMed: 15380510]
- Aikin R, Maysinger D, Rosenberg L. Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. *Endocrinology*. 2004; 145:4522–4531. [PubMed: 15242986]
- Amsterdam A, Tajima K, Sasson R. Cell-specific regulation of apoptosis by glucocorticoids: Implication to their anti-inflammatory action. *Biochem Pharmacol*. 2002; 64:843–850. [PubMed: 12213578]
- Arai T, Hiromatsu K, Kobayashi N, Takano M, Ishida H, Nimura Y, Yoshikai Y. IL-10 is involved in the protective effect of dibutyl cyclic adenosine monophosphate on endotoxin-induced inflammatory liver injury. *J Immunol*. 1995; 155:5743–5749. [PubMed: 7499862]

- Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. *Liver Int.* 2006; 26:1175–1186. [PubMed: 17105582]
- Camargo CA Jr, Madden JF, Gao W, Selvan RS, Clavien PA. Interleukin-6 protects liver against warm ischemia/reperfusion injury and promotes hepatocyte proliferation in the rodent. *Hepatology.* 1997; 26:1513–1520. [PubMed: 9397992]
- Chen Z, Gibson TB, Robinson F, Silvestro L, Pearson G, Xu B, Wright A, Vanderbilt C, Cobb MH. MAP kinases. *Chem Rev.* 2001; 101:2449–2476. [PubMed: 11749383]
- Chen C, Deng M, Sun Q, Loughran P, Billiar TR, Scott MJ. Lipopolysaccharide stimulates p62-dependent autophagy-like aggregate clearance in hepatocytes. *Biomed Res Int.* 2014; 2014:267350. [PubMed: 24683544]
- Cobb MH, Hepler JE, Cheng M, Robbins D. The mitogen-activated protein kinases, ERK1 and ERK2. *Semin Cancer Biol.* 1994; 5:261–268. [PubMed: 7803762]
- Czaja MJ, Ding WX, Donohue TM Jr, Friedman SL, Kim JS, Komatsu M, Lemasters JJ, Lemoine A, Lin JD, Ou JH, Perlmutter DH, Randall G, Ray RB, Tsung A, Yin XM. Functions of autophagy in normal and diseased liver. *Autophagy.* 2013; 9:1131–1158. [PubMed: 23774882]
- Dangi A, Sumpter T, Kimura S, Stolz DB, Murase N, Raimondi G, Vodovotz Y, Huang C, Thomson AW, Gandhi CR. Selective expansion of allogeneic regulatory T cells by hepatic stellate cells: Role of endotoxin and implications for allograft tolerance. *J Immunol.* 2012; 188:3667–3677. [PubMed: 22427640]
- Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell.* 2000; 103:239–252. [PubMed: 11057897]
- Decker K. The response of liver macrophages to inflammatory stimulation. *Keio J Med.* 1998; 47:1–9. [PubMed: 9560526]
- Dhupar R, Klune JR, Evankovich J, Cardinal J, Zhang M, Ross M, Murase N, Geller DA, Billiar TR, Tsung A. Interferon regulatory factor-1 mediates acetylation and release of high mobility group box-1 from hepatocytes during murine liver ischemia-reperfusion injury. *Shock.* 2011; 35:293–301. [PubMed: 20856174]
- Diez-Fernandez C, Sanz N, Alvarez AM, Wolf A, Cascales M. The effect of non-genotoxic carcinogens, Phenobarbital and clofibrate, on the relationship between reactive oxygen species, antioxidant enzyme expression and apoptosis. *Carcinogenesis.* 1998; 19:1715–1722. [PubMed: 9806150]
- Dinant S, Veteläinen RL, Florquin S, van Vliet AK, van Gulik TM. IL-10 attenuates hepatic I/R injury and promotes hepatocyte proliferation. *J Surg Res.* 2007; 141:176–182. [PubMed: 17543989]
- Ding WX, Yin XM. Dissection of the multiple mechanisms of TNF- α -induced apoptosis in liver injury. *J Cell Mol Med.* 2004; 8:445–454. [PubMed: 15601573]
- Farges MC, Vasson MP, Davot P, Villie F, Meunier MT, Marcollet M, Cynober L. Supplementation of oral nutrition with pancreatic enzymes improves the nutritional status of aged endotoxemic rats. *Nutrition.* 1996; 112:189–194.
- Fausto N, Laird AD, Webber EM. Role of growth factors and cytokines in hepatic regeneration. *FASEB J.* 1995; 9:1527–1536. [PubMed: 8529831]
- Fukui H. Relation of endotoxin, endotoxin binding proteins and macrophages to severe alcoholic liver injury and multiple organ failure. *Alcohol Clin Exp Res.* 2005; 29:172S–179S. [PubMed: 16344605]
- Gandhi CR, Kuddus RH, Nemoto EM, Murase N. Endotoxin treatment causes up-regulation of endothelin system in the liver: Amelioration of increased portal resistance by endothelin receptor antagonism. *J Gastroenterol Hepatol.* 2001; 6:61–69.
- Gandhi, CR. Hepatic Stellate Cells. Monga, SP., editor. Springer: Molecular Pathology of Liver Diseases; 2010. p. 53-80.
- Geller DA, Nguyen D, Shapiro RA, Nussler A, Di SM, Freeswick P, Wang SC, Tweardy DJ, Simmons RL, Billiar TR. Cytokine induction of interferon regulatory factor-1 in hepatocytes. *Surgery.* 1993; 114:235–242. [PubMed: 7688150]
- Geller DA, Freeswick PD, Nguyen D, Nussler AK, Di Silvio M, Shapiro RA, Wang SC, Simmons RL, Billiar TR. Differential induction of nitric oxide synthase in hepatocytes during endotoxemia and the acute-phase response. *Arch Surg.* 1994; 129:165–171. [PubMed: 7508220]

- Gotoh T, Mori M. Nitric oxide and endoplasmic reticulum stress. *Arterioscler Thromb Vasc Biol.* 2006; 26:1439–1446. [PubMed: 16645155]
- Gunawan BK, Liu ZX, Han D, Hanawa N, Gaarde WA, Kaplowitz N. C-Jun N-terminal kinase plays a major role in murine acetaminophen hepatotoxicity. *Gastroenterology.* 2006; 131:165–178. [PubMed: 16831600]
- Hasegawa, D., Wallace, MC., Friedman, SL. Stellate cells and hepatic fibrosis. In: Gandhi, CR., Pinzani, M., editors. *Stellate cells in health and disease.* London: Elsevier; 2015. p. 41-62.
- Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. *Nature.* 2006; 441:885–889. [PubMed: 16625204]
- Harris J. Autophagy and cytokines. *Cytokine.* 2011; 56:140–144. [PubMed: 21889357]
- Harvey SAK, Dangi A, Tandon A, Gandhi CR. The transcriptomic response of rat hepatic stellate cells to endotoxin: Implications for hepatic inflammation and immune regulation. *PLoS ONE.* 2013; 8:e82159. [PubMed: 24349206]
- Hers I, Vincent EE, Tavaré JM. Akt signalling in health and disease. *Cell Signal.* 2011; 23:1515–1527. [PubMed: 21620960]
- Jia G, Kong R, Ma ZB, Han B, Wang YW, Pan SH, Li YH, Sun B. The activation of c-Jun NH2-terminal kinase is required for dihydroartemisinin-induced autophagy in pancreatic cancer cells. *J Exp Clin Cancer Res.* 2014; 33:8. [PubMed: 24438216]
- Kaniuk NA, Kiraly M, Bates H, Vranic M, Volchuk A, Brumell JH. Ubiquitinated-protein aggregates form in pancreatic b-cells during diabetes-induced oxidative stress and are regulated by autophagy. *Diabetes.* 2007; 56:930–939. [PubMed: 17395740]
- Kim AD, Kang KA, Kim HS, Kim DH, Choi YH, Lee SJ, Kim HS, Hyun JW. A ginseng metabolite, compound K, induces autophagy and apoptosis via generation of reactive oxygen species and activation of JNK in human colon cancer cells. *Cell Death Dis.* 2013; 4:e750. [PubMed: 23907464]
- Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K. Loss of autophagy in the central nervous system causes neurodegeneration in mice. *Nature.* 2006; 441:880–884. [PubMed: 16625205]
- Komatsu M, Wang QJ, Holstein GR, Friedrich VL Jr, Iwata J, Kominami E, Chait BT, Tanaka K, Yue Z. Essential role for autophagy protein Atg7 in the maintenance of axonal homeostasis and the prevention of axonal degeneration. *Proc Natl Acad Sci USA.* 2007; 104:14489–14494. [PubMed: 17726112]
- Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, et al. Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. *Autophagy.* 2008; 4:151–175. [PubMed: 18188003]
- Li JQ, Qi HZ, He ZJ, Hu W, Si ZZ, Li YN, Li DB. Cytoprotective effects of human interleukin-10 gene transfer against necrosis and apoptosis induced by hepatic cold ischemia/reperfusion injury. *J Surg Res.* 2009; 157:e71–e78. [PubMed: 19555976]
- Lorin S, Pierron G, Ryan KM, Codogno P, Djavaheri-Mergny M. Evidence for the interplay between JNK and p53-DRAM signalling pathways in the regulation of autophagy. *Autophagy.* 2010; 6:153–154. [PubMed: 19949306]
- Ma Y, Hendershot LM. The mammalian endoplasmic reticulum as a sensor for cellular stress. *Cell Stress Chaperones.* 2002; 7:222–229. [PubMed: 12380691]
- Maddox JF, Amuzie CJ, Li M, Newport SW, Sparkenbaugh E, Cuff CF, Pestka JJ, Cantor GH, Roth RA, Ganey PE. Bacterial- and viral-induced inflammation increases sensitivity to acetaminophen hepatotoxicity. *J Toxicol Environ Health A.* 2010; 73:58–73. [PubMed: 19953420]
- Martinovi V, Grigorov I, Bogoevi D, Petrovi A, Jovanovi S, Ili M, Ivanovi M, Mati S. Activation level of JNK and Akt/ERK signaling pathways determinates extent of DNA damage in the liver of diabetic rats. *Cell Physiol Biochem.* 2012; 30:723–734. [PubMed: 22854746]
- McMillian M, Nie A, Parker JB, Leone A, Kemmerer M, Bryant S, Herlich J, Yieh L, Bittner A, Liu X, Wan J, Johnson MD, Lord P. Drug-induced oxidative stress in rat liver from a toxicogenomics perspective. *Toxicol Appl Pharmacol.* 2005; 207:171–178. [PubMed: 15982685]

- Michalopoulos GK, DeFrances MC. Liver regeneration. *Science*. 1997; 276:60–66. [PubMed: 9082986]
- Mizushima N, Yoshimori T, Levine B. Methods in mammalian autophagy research. *Cell*. 2010; 140:313–326. [PubMed: 20144757]
- Ni HM, Chen X, Ding WX, Schuchmann M, Yin XM. Differential roles of JNK in ConA/GalN and ConA-induced liver injury in mice. *Am J Pathol*. 2008; 173:962–972. [PubMed: 18772342]
- Ni HM, Bockus A, Boggess N, Jaeschke H, Ding WX. Activation of autophagy protects against acetaminophen-induced hepatotoxicity. *Hepatology*. 2012; 55:222–232. [PubMed: 21932416]
- Pearson G, Robinson F, Beers-Gibson T, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocr Rev*. 2001; 22:153–183. [PubMed: 11294822]
- Puche JE, Saiman Y, Friedman SL. Hepatic stellate cells and liver fibrosis. *Compr Physiol*. 2013; 3:1473–1492. [PubMed: 24265236]
- Qiao L, Han SI, Fang Y, Park JS, Gupta S, Gilfor D, Amorino G, Valerie K, Sealy L, Engelhardt JF, Grant S, Hylemon PB, Dent P. Bile acid regulation of C/EBPbeta, CREB, and c-Jun function, via the extracellular signal-regulated kinase and c-Jun NH2-terminal kinase pathways, modulates the apoptotic response of hepatocytes. *Mol Cell Biol*. 2003; 23:3052–3066. [PubMed: 12697808]
- Raciti M, Lotti LV, Valia S, Pulcinelli FM, Di Renzo L. JNK2 is activated during ER stress and promotes cell survival. *Cell Death Dis*. 2012; 3:e429. [PubMed: 23171849]
- Roberts RA, James NH, Cosulich SC. The role of protein kinase B and mitogen-activated protein kinase in epidermal growth factor and tumor necrosis factor alpha-mediated rat hepatocyte survival and apoptosis. *Hepatology*. 2000; 31:420–427. [PubMed: 10655266]
- Sabapathy K, Hochedlinger K, Nam SY, Bauer A, Karin M, Wagner EF. Distinct roles for JNK1 and JNK2 in regulating JNK activity and c-Jun-dependent cell proliferation. *Mol Cell*. 2004; 15:713–725. [PubMed: 15350216]
- Sah SP, Tirkey N, Kuhad A, Chopra K. Effect of quercetin on lipopolysaccharide induced-sickness behavior and oxidative stress in rats. *Indian J Pharmacol*. 2011; 43:192–196. [PubMed: 21572657]
- Santiago-Lomeli M, Gómez-Quiroz LE, Ortíz-Ortega VM, Kershenovich D, Gutiérrez-Ruiz MC. Differential effect of interleukin-10 on hepatocyte apoptosis. *Life Sci*. 2005; 76:2569–2579. [PubMed: 15769481]
- Schmeisser H, Fey SB, Horowitz J, Fischer ER, Balinsky CA, Miyake K, Bekisz J, Snow AL, Zoon KC. Type I interferons induce autophagy in certain human cancer cell lines. *Autophagy*. 2013; 9:683–696. [PubMed: 23419269]
- Schmeisser H, Bekisz J, Zoon KC. New Function of Type I IFN: Induction of autophagy. *J Interferon Cytokine Res*. 2014; 34:71–78. [PubMed: 24428799]
- Schwabe RF, Brenner D. Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: Role of IKK, JNK, and ROS pathways. *Am J Physiol Gastrointest Liver Physiol*. 2006; 290:G583–G589. [PubMed: 16537970]
- Speicher T, Köhler UA, Choukér A, Werner S, Weiland T, Wendel A. Fructose protects murine hepatocytes from tumor necrosis factor-induced apoptosis by modulating JNK signaling. *J Biol Chem*. 2012; 287:1837–1846. [PubMed: 22086922]
- Stewart R, Dangi A, Huang C, Murase N, Kimura S, Stolz DB, Wilson GC, Lentsch AB, Gandhi CR. A novel mouse model of depletion of stellate cells clarifies their role in ischemia/reperfusion- and endotoxin-induced acute liver injury. *J Hepatol*. 2014; 60:298–305. [PubMed: 24060854]
- Strozyk E, Kulms D. NFκB: Cell survival or cell death? *Signal Transduction*. 2005; 6:334–339.
- Szeto J, Kaniuk NA, Canadien V, Nisman R, Mizushima N, Yoshimori T, Bazett-Jones DP, Brumell JH. ALIS are stress-induced protein storage compartments for substrates of the proteasome and autophagy. *Autophagy*. 2006; 2:189–199. [PubMed: 16874109]
- Thirunavukkarasu C, Uemura T, Wang LF, Watkins SC, Gandhi CR. Normal rat hepatic stellate cells respond to endotoxin in LBP-independent manner to produce inhibitor(s) of DNA synthesis in hepatocytes. *J Cell Physiol*. 2005; 204:654–665. [PubMed: 15828022]
- Thirunavukkarasu C, Watkins S, Gandhi CR. Mechanisms of endotoxin-induced nitric oxide, interleukin-6 and tumor necrosis factor-α production in activated rat hepatic stellate cells: Role of p38MAPK. *Hepatology*. 2006; 44:389–398. [PubMed: 16871588]

- Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science*. 2000; 288:870–874. [PubMed: 10797012]
- Tsung A, Stang MT, Ikeda A, Critchlow ND, Izuishi K, Nakao A, Chan MH, Jeyabalan G, Yim JH, Geller DA. The transcription factor interferon regulatory factor-1 mediates liver damage during ischemia-reperfusion injury. *Am J Physiol Gastrointest Liver Physiol*. 2006; 290:G1261–G1268. [PubMed: 16410367]
- Uemura T, Gandhi CR. Inhibition of DNA synthesis in cultured hepatocytes by endotoxin-conditioned medium of activated stellate cells is transforming growth factor- β - and nitric oxide-independent. *Br J Pharmacol*. 2001; 133:1125–1133. [PubMed: 11487524]
- Wang QJ, Ding Y, Kohtz DS, Mizushima N, Cristea IM, Rout MP, Chait BT, Zhong Y, Heintz N, Yue Z. Induction of autophagy in axonal dystrophy and degeneration. *J Neurosci*. 2006; 26:8057–8068. [PubMed: 16885219]

Figure 1.

Transmission electron micrograph showing LPS/HSC-induced autophagy in hepatocytes. A. Hepatocytes were incubated in control medium (HC-CT), medium containing 100 ng/mL LPS (HC-LPS) or medium conditioned by HSCs without (HSC to HC) or with LPS (LPS/HSC to HC) for 6 h. Hepatocytes incubated with LPS (b) or HSC-conditioned medium (c) are ultrastructurally similar to control cells (a). Several autophagosomes (asterisks) are seen in LPS/HSC-treated hepatocytes (d); autophagosomes contained glycogen and other cellular material (e and f), mitochondria (g and h), and ER (h). Scale bar: 2 μm (a–d) and 500nm (e–h). B. Bar graph shows the number of autophagy vesicles per hepatocyte counted in at least 3 sections ± SD.

Figure 2.

LPS/HSC increase LC3-II expression in hepatocytes (A) Hepatocytes (HC) were incubated in control, LPS, HSC or LPS/HSC medium for 6 h, and analyzed for LC3-II localization via immunofluorescence. Bar graphs show percentage of hepatocytes with LC3-II accumulation counted in 7–10 fields/well, and the number of LC3-II puncta per 10 hepatocytes. (B) Hepatocytes were incubated in LPS/HSC medium for indicated times, or (C) preincubated in medium containing 1 nM baflomycin (BAF) or 1 mM 3-methyladenine (3-MA) for 15 min, then placed in indicated media±baflomycin or 3-MA for 6 h (right panel). Western analysis was performed to assess LC3-II formation and p62 expression. Bar graphs show

densitometric quantification of the indicated molecules normalized to the intensity of β -actin.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 3.

Blockade of autophagy increases LPS/HSC-induced apoptosis of hepatocytes. Hepatocytes were incubated in control (HC-CT), LPS (HC-LPS), HSC (HSC to HC) or LPS/HSC (LPS/HSC to HC) medium $\pm$ 1 mM 3-MA for 24 h. Images show TUNEL-positive hepatocytes. Bar graph shows percentage of TUNEL-positive hepatocytes (averages of 3 experiments $\pm$ SD).

Figure 4.

LPS/HSC-induced activation of JNK mediates caspase-3 activation and autophagy in hepatocytes. (A) Western blot shows JNK or AKT activation in hepatocytes incubated in control, LPS, HSC or LPS/HSC medium for 6 h. Bar graph shows ratio of densitometry values for P-JNK or P-AKT vs β-actin. (B,C) Hepatocytes were pretreated with 10 μM SP600125 (15 min) then incubated in LPS or LPS/HSC medium ± SP600125 for 6 h (B) or 24 h (C) before Western analysis and enzymatic activity for caspase-3 and Western analysis for JNK activation, or TUNEL staining were performed. (D) Western blot shows LC3-II in

hepatocytes incubated with SP600125 (15 min), then in LPS/ HSC medium $\pm$ SP600125 (6h). Bar graph shows LC3-II/ β -actin ratio (n = 3–4).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

IFN β mediates LPS/HSC-induced JNK activation in hepatocytes. (A) Bar graph shows IFN β mRNA expression in HSCs incubated with LPS for indicated times. Immunostaining shows intracellular IFN β . Upper panel—Green: α -sma; red:IFN β ; blue:nuclei. Lower panel— images in red channel. (B) Hepatocytes were incubated in LPS/HSC medium or 200 U/mL IFN β or (C) in LPS or LPS/HSC medium $\pm$ anti-IFN β -Ab (1.5 μ g/mL) for 6 h. Western blot shows LC3-II. Bar graphs shows average of 3 determinations (LC3-II/ β -actin) $\pm$ SD. (D) Hepatocytes were incubated with 200U/mL IFN β (left panel), or LPS- or LPS/HSC medium $\pm$ anti-IFN β -Ab (right panel) for 6 h; Western blot shows JNK activation.

Figure 6.

IFN β -induced inhibition of DNA synthesis, increased caspase-3 activation and apoptosis of hepatocytes. (A,B) Hepatocytes were incubated with 200 U/ml IFN β for 24 h (A, C) or 6 h (B). DNA synthesis (A), caspase-3 activation (B) and number of apoptotic cells (TUNEL staining) (C) were determined. Values show averages of triplicate determinations $\pm$ SD. (D) TUNEL staining of hepatocytes incubated for 1 h with anti-IFN β -Ab (1.5 μ g/mL) or vehicle, then with control or HSC-conditioned media $\pm$ anti-IFN β -Ab for 24 h. Graph shows quantification of TUNEL-positive cells.

Figure 7.

Apoptosis and autophagy in LPS-treated rat liver. (A) H/E-sections show PBS-treated control liver with normal histology (a), and mild inflammation and sinusoidal dilation in LPS-treated liver (b). (c,d) Confocal images–TUNEL-positive (red) hepatocytes away from (thick arrow) and in proximity (arrowheads) of desmin-positive (green) HSCs. (B) TEM-rough endoplasmic reticulum (ER) in control (a,c) is dilated (dER) with loss of ribosomes (long arrows) upon LPS treatment (b,d). Cytosolic vacuoles (short arrows) and autophagosomes (asterisks) containing mitochondria (e) and cellular debris (f). Scale bar=500 nm. (C) Western blot of hepatocytes from LPS-treated rat livers (n = 4) shows JNK

and caspase-3 activation, and increased LC3-II, P-PERK, P-eIF2 α and CHOP. (D) mRNA expression of IFN β in LPS-treated rat livers.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 8.

LPS-induced LC3-II formation in HSC-depleted mice. Protein lysates of the livers of HSC-sufficient and HSC-depleted mice were prepared 6h after administration of 10 mg/kg LPS. Detailed procedures of HSC depletion and LPS-induced injury are described (Stewart et al., 2014). The lysates were subjected to Western analysis to assess LC3-II formation.