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RESEARCH ARTICLE

Translational Physiology

Distinct responses between healthy and cirrhotic human livers upon lipopolysaccharide challenge: possible implications for acute-on-chronic liver failure

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Abstract

Patients with acute-on-chronic liver failure (ACLF) are at risk of developing acute hepatic decompensation and organ failures with an unraveled complex mechanism. An altered immune response toward insults in cirrhotic compared with healthy livers may contribute to the ACLF development. Therefore, we aim to investigate the differences in inflammatory responses between cirrhotic and healthy livers using human precision-cut liver slices (PCLSs) upon the lipopolysaccharide (LPS) challenge. PCLSs prepared from livers of patients with cirrhosis or healthy donors of liver transplantation were incubated ex vivo with or without LPS for up to 48 h. Viability test, qRT-PCR, and multiplex cytokine assay were performed. Regulation of the LPS receptors during incubation or with LPS challenge differed between healthy versus cirrhotic PCLSs. LPS upregulated *TLR-2* in healthy PCLSs solely ($P < 0.01$). Culturing for 48 h induced a stronger inflammatory response in the cirrhotic than healthy PCLS. Upon LPS stimulation, cirrhotic PCLSs secreted more proinflammatory cytokines (IL-8, IL-6, TNF-α, eotaxin, and VEGF) significantly and less anti-inflammatory cytokine (IL-1ra) than those of healthy. In summary, cirrhotic PCLSs released more proinflammatory and less anti-inflammatory cytokines after LPS stimuli than healthy, leading to dysregulated inflammatory response. These events could possibly resemble the liver immune response in ACLF.

NEW & NOTEWORTHY Precision-cut liver slices (PCLSs) model provides a unique platform to investigate the different immune responses of healthy versus cirrhotic livers in humans. Our data show that cirrhotic PCLSs exhibit excessive inflammatory response accompanied by a lower anti-inflammatory cytokine release in response to LPS; a better understanding of this alteration may guide the novel therapeutic approaches to mitigate the excessive inflammation during the onset of acute-on-chronic liver failure.

acute-on-chronic liver failure; cirrhosis; human liver; lipopolysaccharide; precision-cut liver slices

INTRODUCTION

Acute-on-chronic liver failure (ACLF) is a syndrome caused by infectious or noninfectious insults upon a preexisting chronic liver disease. It is characterized by acute hepatic decompensation and a high short-term mortality rate (death within 28 days after hospitalization), often accompanied by failing organ(s) (1–3). Currently, there is no standard treatment for ACLF except for organ support and treatment of associated complications, such as antimicrobial treatment or hemorrhage management. Liver transplantation is a possibility, however, organ shortage and high contraindication rate in the patients with ACLF often hamper this treatment opportunity (2). Therefore, more insight into the pathology of ACLF is needed to develop new treatment strategies, including pharmacological ones.

The pathophysiology of ACLF is largely unknown. Up to 40% of ACLF cases have no traceable trigger (2). In other cases, the most common triggers of ACLF are infection and alcoholic hepatitis (4, 5). During infection, pathogen-

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associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), from bacterial infections activate the pattern recognition receptors (PRRs) of the liver innate immune system, causing and exaggerating hepatic inflammation (6). In alcoholic hepatitis, PAMPs of the gut microbiota reaches the liver through systemic circulation as well as damage-associated molecular patterns (DAMPs) derived from necrotic hepatocytes would exacerbate the liver inflammation through activation of the PRRs (6, 7). Accordingly, dysregulated systemic inflammation during infection or alcoholic hepatitis possibly drives the progression of ACLF and correlates positively to the severity and poor outcome of the syndrome (8).

Liver is a frontline immunological organ that counterpoises pro- and anti-inflammation, balancing the resistance and tolerance to the stimulus (9). The overall aim of ACLF treatment is to prevent further damage to the liver during the acute phase and thereby create a prosperous environment for regeneration of the liver to its original cirrhotic state. Thus, studying the different responses between the cirrhotic versus healthy liver toward stimuli would provide useful information on the pathology of ACLF. So far, there is no study comparing the inflammatory response of cirrhotic versus healthy human liver toward an acute LPS challenge at the tissue level.

Basic experimental research in the laboratory on the molecular mechanism of ACLF often relies on animal models or in vitro experiments that utilizes often single-cell cultures isolated from healthy individuals or patients with diseases (10–13). Specifically, acute insults (induced by D-galactosamine combined with LPS or bacterial infection) upon chronic liver injury (induced by carbon tetrachloride injection or bile duct ligation) is the main strategy to develop ACLF in these animals (10–13). However, these studies normally use healthy rodents that might not fully represent the complex situation in patients with cirrhosis. In addition, the extrapolation of data from animal to human is not always reliable.

Cell culture studies showed that LPS-treated monocytes or peripheral blood mononuclear cells derived from patients with cirrhosis secreted more TNF-α, IL-1β, IL-6, and IL-8 than that of healthy controls (14–16). Furthermore, patients with cirrhosis with bacterial infections showed excessive production of TNF-α and IL-6 in the serum, which is positively associated with organ failures and mortality (14). Unfortunately, none of these studies showed the different responses of healthy versus cirrhotic liver to the LPS stimuli at an organ level.

Precision-cut liver slices (PCLSs) are a unique ex vivo model to investigate LPS-induced inflammation using fresh metabolizing tissue from rodents or humans, as previously reported (17–20). Using PCLS from patients with cirrhosis would be the closest to the clinical situation of actual human subjects and challenging these slices with LPS might be a valuable new model to mimic liver inflammation during ACLF situations. Thus, the current study aimed to investigate the differences between healthy versus cirrhotic livers in response to an LPS challenge using healthy and cirrhotic human PCLS, providing additional insights for future mechanistic studies with regard to ACLF treatment.

MATERIALS AND METHODS

Ethical Consideration and Obtaining of Human Liver Tissue

The use of human tissue was approved by the Medical Ethical Committee of the University Medical Center Groningen (UMCG), according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research (www.federa.org), refraining the need of written consent for "further use" of coded-anonymous human tissue. The procedures were carried out in accordance with the experimental protocols approved by the Medical Ethical Committee of the UMCG. Surgical excess material of donor livers was characterized as clinically healthy liver (n = 7). Explanted cirrhotic liver of clinically diagnosed patients with end-stage liver disease undergoing liver transplantation was characterized as cirrhotic liver (n = 6). Patient demographics are shown in Supplemental Table S1.

Preparation of the Precision-Cut Liver Slices

Precision-cut human liver slices were prepared as previously described (21). In brief, surgically excess human liver was obtained and cylindrical cores were made using a 6-mm biopsy punch and preserved in ice-cold University of Wisconsin (UW) tissue preservation solution (DuPont Critical Care, Waukegan, IL) until slicing. Krebs-Henseleit buffer was supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM $NaHCO_3$ (Merck), and 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (MP Biomedicals, Aurora, OH), saturated with carbogen (95% O_2 and 5% CO_2), and used as slicing solution. The size of the PCLSs was adjusted by the wet weight (4–5 mg) to a thickness of around 250 μm.

Incubation of the Precision-Cut Liver Slices

Before incubation, the PCLSs were collected as 0-h samples. Precision-cut human liver slices were incubated as previously described (21). William's E medium with GlutaMAX (Life Technologies, Carlsbad) 2.75 g/mL D-glucose monohydrate (Merck, Darmstadt, Germany), 50 μg/mL gentamicin (Invitrogen, Paisley, UK) was prepared as 1.3 mL/well in 12-well plates, preheated and oxygenized in the incubator at 37°C with a continuous 5% CO_2–80% O_2 supply for at least 30 min before plating the PCLSs. The PCLSs were incubated individually for 1 h as preincubation in the culture medium. After preincubation, slices were changed to preheated and oxygenized fresh medium or medium supplemented with 5 μg/mL (5,000 EU/mL) ultrapure LPS from *Escherichia coli* O111:B4 (InvivoGen, Toulouse, France) and the medium was refreshed at 24 h. Slices were further incubated with or without LPS until 48 h and collected for further analysis. Three slices were incubated for each condition.

Assessment of Viability of the Precision-Cut Liver Slices

After incubation, slices were collected individually in a 1.5-mL Eppendorf safe-lock tube with minibeads and 1 mL sonification solution, snap frozen in liquid nitrogen, and stored at −80°C until analysis. ATP content of each slice was determined using ATP bioluminescence assay kit class II

(Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction and as described previously (21).

Quantitative Real-Time Polymerase Chain Reaction

After incubation, the triplicate slices were collected in a 1.5-mL Eppendorf safe-lock tube with minibeads, snap-frozen in liquid nitrogen, and stored at −80°C until analysis (0-h samples were collected before incubation). Total RNA was extracted using FavorPrep tissue total RNA minikit (FAVORGEN Biotech Corp, Vienna, Austria), according to the manufacturer's instruction. Concentration and purity of the RNA were determined using Synergy HT (Biotek, Swindon, UK) at wavelength of 260/280, a value between 1.9 and 2.1 was considered of good quality of RNA and stored at −80°C. cDNA was reverse transcripted from 1 μg total RNA using Reverse Transcription Kit (Promega, Leiden, the Netherlands) at 22°C for 10 min, 42°C for 15 min, 95°C for 5 min, and stored at −20°C. Gene expression level was assessed by quantitative real-time polymerase chain reaction in ViiA 7 Real-Time PCR System with a FastStart Universal SYBR Green Master (ROX) or FastStart Universal Probe Master (ROX) (Roche Diagnostics, Mannheim, Germany) plus primer pairs (Supplemental Table S2). The experimental condition of SYBR and Taqman mix is shown in Supplemental Tables S3 and S4. Relative expression values were expressed as percentages compared with housekeeping genes (100%): glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Taqman) and 18S (SYBR).

Bio-Plex Pro Human Inflammation Assay and ELISA

Culture medium was pooled together from three wells of the same treatment, and stored at −80°C for further analysis. Cytokines in the culture medium were tested with Bio-Plex Pro Human Cytokine Grp I panel (27-Plex) according to the manufacturer's protocol (Bio-Rad, Winninglaan, Belgium). The culture medium was centrifuged at 13,000 rpm for 5 min, the supernatant was diluted four times with new culture medium before the test. MAGPIX multiplexing instrument (Luminex, Austin, TX) was used to detect the mean fluorescent intensity (MFI) of each sample. The Concentration of cytokines was calculated from the respective standard curve. Concentration of high mobility group protein 1 (HMGB1) was measured using the Human HMGB1/HMG-1 ELISA Kit (Colorimetric, Novus Biologicals) according to the manufacturer's protocol. The concentration (pg/mL) of the cytokine or HMGB1 was normalized with the respective protein content of the slice (μg).

Morphology

Slices were fixed in 4% buffered formalin for 24 h, transferred to 70% ethanol, dehydrated, embedded in paraffin, and sectioned (4 μm). Hematoxylin and eosin (H&E) or Sirius red staining was performed after deparaffinizing and rehydrating the sections.

Statistical Analysis

The data were shown as means ± SE. The difference between healthy versus cirrhotic was compared using Mann–Whitney test; difference within the healthy or cirrhotic groups (with or without LPS treatment) was compared using Kruskal–Wallis test followed by Dunn's multiple comparisons test with GraphPad Prism v. 6.0. A P value of <0.05 was considered to be significant.

RESULTS

Viability and Morphology of the Human PCLS

To evaluate the viability of the slices during incubation or treatment, we assessed the ATP content of the slices. During 48 h of incubation, both healthy and cirrhotic human PCLSs remained viable (Fig. 1, *A* and *B*), and LPS did not significantly influence the viability of the healthy and cirrhotic PCLS (Fig. 1*C*). During 48-h incubation, the morphology of the healthy PCLSs showed intact hepatocytes and the presence of other nonparenchymal cells across the slice tissue with their natural context preserved and no signs of cell death; morphology of cirrhotic PCLSs maintained the same as before incubation (Fig. 1*D*). LPS did not significantly impair the morphology of both healthy and cirrhotic PCLS compared with the respective control (Supplemental Figs. S1 and S2).

Expression and Regulation of LPS Receptors in the Human PCLS

We continued to test the gene expression of a variety of LPS receptors and coreceptors using qRT-PCR, aiming to confirm the presence of this response system in the PCLS incubations. For this, Toll-like receptor-4 (TLR-4), the major receptor for LPS, coreceptors such as lymphocyte antigen 96 (MD-2), CD180 (RP105), lymphocyte antigen 86 (MD-1), CD14, and TLR-2 were tested. Before incubation (0 h), *RP105* and *MD-1* were significantly higher expressed in freshly prepared cirrhotic than healthy PCLSs (Fig. 2*A*, Supplemental Fig. S3*A*). During incubation, *TLR-4* and *MD-2* gene expression was markedly augmented in both healthy and cirrhotic PCLS at 48 h. Moreover, *CD14, RP105*, and *MD-1* were significantly induced only in healthy at 24 h and 48 h, whereas *TLR-2* was upregulated only in cirrhotic at 48 h (Fig. 2*B*). After LPS treatment, *TLR-4* and *MD-1* were downregulated in both healthy (*TLR-4* at 24 h and *MD-1* at 24 h and 48 h) and cirrhotic (*TLR-4* at 48 h and *MD-1* at 24 h and 48 h) PCLSs, whereas *TLR-2* was strikingly upregulated solely in healthy PCLS at both 24 h and 48 h ($P < 0.01$); *MD-2*, however, was slightly downregulated in the cirrhotic PCLSs only at 48 h ($P < 0.05$). CD14 was not differently expressed between the healthy versus cirrhotic PCLSs at baseline, not influenced by LPS challenge, but was elevated during incubation at 24 h solely in the healthy PCLSs. Interestingly, *RP105* was regulated in the opposite directions by LPS: down in healthy and up in cirrhotic PCLS at 24 h. (Fig. 2*C*). Taken together, the basal expression levels differed between healthy and cirrhotic PCLSs; all receptors are present and dynamically and differently regulated in the ex vivo PCLS system during incubation and after the LPS challenge (Fig. 2*D*).

Inflammatory Response in the Human PCLS before and during Incubation

We next examined the impact of incubation on the inflammatory response of the PCLSs by qRT-PCR or Bio-Plex assay. Before incubation, among the tested genes, only *IL-8* mRNA of cirrhotic PCLSs was relatively higher than that of healthy PCLSs (Fig. 3*A*, Supplemental Fig. S3*B*). During incubation,

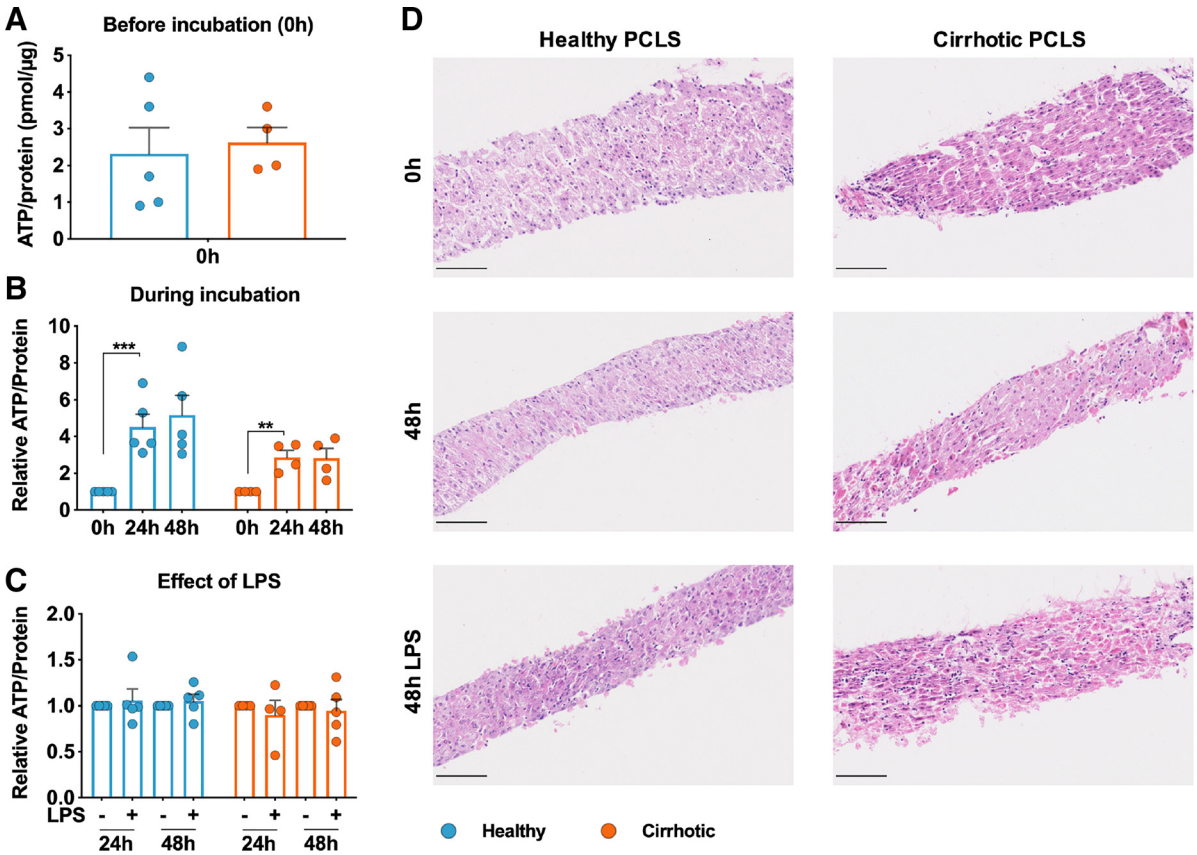


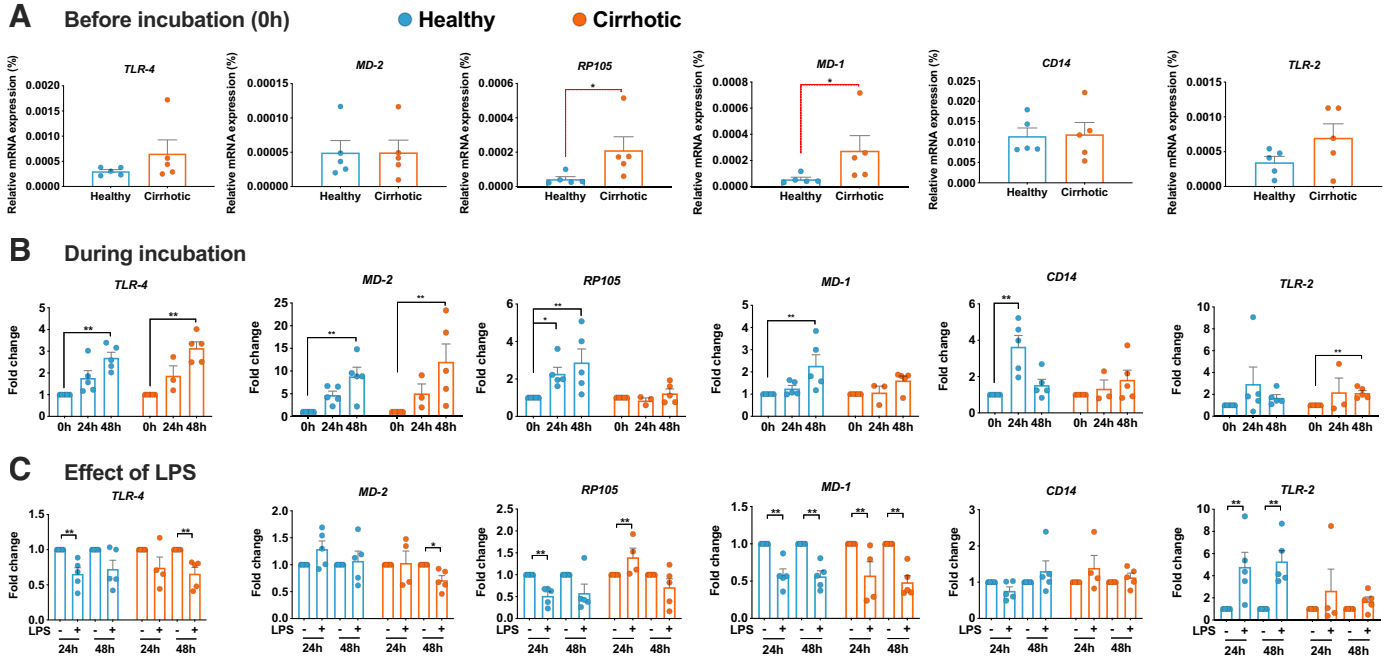
Figure 1. Viability and morphology of human healthy and cirrhotic precision-cut liver slices (PCLSs). Viability of healthy and cirrhotic PCLSs before incubation (ATP/protein ratio) (*A*); during 48-h incubation (relative increase compared with the respective 0 h) (*B*); and with or without the treatment of LPS (relative increase compared with the respective 24 h or 48 h) (*C*). Data are means ± SE; healthy: $n = 5$, cirrhotic: $n = 4$. $^{**}P < 0.01$, $^{***}P < 0.001$. *D*: morphology of human healthy and cirrhotic precision-cut liver slices, H&E staining of PCLSs after incubation for 48 h with or without LPS (5 µg/mL) treatment. Representative images of healthy: $n = 3$; cirrhotic: $n = 3$ livers. Scale bars: 100 µM. H&E, hematoxylin and eosin; LPS, lipopolysaccharide.

there was a spontaneous onset of inflammatory response in both healthy and cirrhotic PCLSs, indicated by mRNA upregulation of the proinflammatory genes *IL-8*, *IL-6*, *IL-1β*, and *TNF-α,* among which *IL-8* (24 h and 48 h), *IL-6* (24 h), and *IL-1β* (24 h) were upregulated to a higher level in cirrhotic PCLSs than in healthy (Fig. 3*B*). Of note, although the actual value is higher in cirrhotic PCLSs, the fold change compared with the respective 0 h did not significantly differ between cirrhotic versus healthy during 24 h or 48 h incubation, except for *IL-1β*, which appeared to be higher in cirrhotic PCLSs at 24 h (Fig. 3, *C* and *D*). Moreover, at the protein level, IL-8, IFN-γ, IL-15, VEGF, MCP-1, and IL-4 were secreted from cirrhotic PCLSs in a significantly higher level when compared with those of healthy PCLSs at 24 h (Fig. 4). Taken together, these data show that spontaneous inflammatory response is stronger in the cirrhotic PCLSs than in healthy ones during incubation.

Inflammatory Response in the Human Precision-Cut Liver Slices after LPS Challenge

To further explore the altered immune response of cirrhotic PCLSs compared with healthy ones, we challenged the PCLSs with LPS and examined the inflammatory markers on gene and protein levels. At the gene expression level, LPS upregulated *IL-8*, *IL-6*, and *IL-1β* mRNA expression both in healthy and cirrhotic PCLSs at 24 h and 48 h (except for IL-6 which is only at 24 h in cirrhotic) (Fig. 5*A*); as expected, higher levels of *IL-8* and *IL-6* were observed in cirrhotic PCLSs after LPS treatment at 24 h (Fig. 5*A*). In contrast, LPS-induced fold change of IL-1β gene expression was lower in cirrhotic PCLSs than in healthy (Fig. 5, *B* and *C*).

Next, we investigated whether LPS induced inflammatory response on protein level with Bio-Plex assay. Cirrhotic PCLSs secreted higher amounts of proinflammatory cytokines (IL-8, IL-6, and VEGF at 24 h; TNF-α and eotaxin at 48 h) when compared with healthy PCLSs (Fig. 6*A*). However, cirrhotic PCLSs secreted lower levels of MIP-1β (48 h) and IL-1ra (anti-inflammatory cytokine, at 24 h and 48 h) than the healthy (Fig. 6*B*). In accordance with the gene expression results, although the actual concentrations of cytokines in the supernatants of cirrhotic PCLSs were higher, the relative increment of inflammatory cytokines (IL-2, IL-4, IL-15, eotaxin, IFN-γ, and MCP-1 at 24 h; IL-4 and eotaxin at 48 h) by LPS was lower in cirrhotic than healthy PCLSs (Fig. 7, Supplemental Table S5). In addition, other cytokines that did not differ between cirrhotic and healthy with LPS treatment were listed in Supplemental Fig. S4. Collectively, cirrhotic PCLSs secreted more proinflammatory and less IL-1ra (anti-inflammatory cytokine) than the healthy ones.



D Summary table

	Only in healthy	Only in cirrhotic	In both
Higher before incubation	-	*RP105, MD-1*	N.A.
Regulated during incubation	*RP105, MD-1,CD14*	*TLR-2*	*TLR-4, MD-2*
Regulated by LPS	*TLR-2*	*MD-2*	*TLR-4, MD-1, **RP105***

upregulated
downregulated

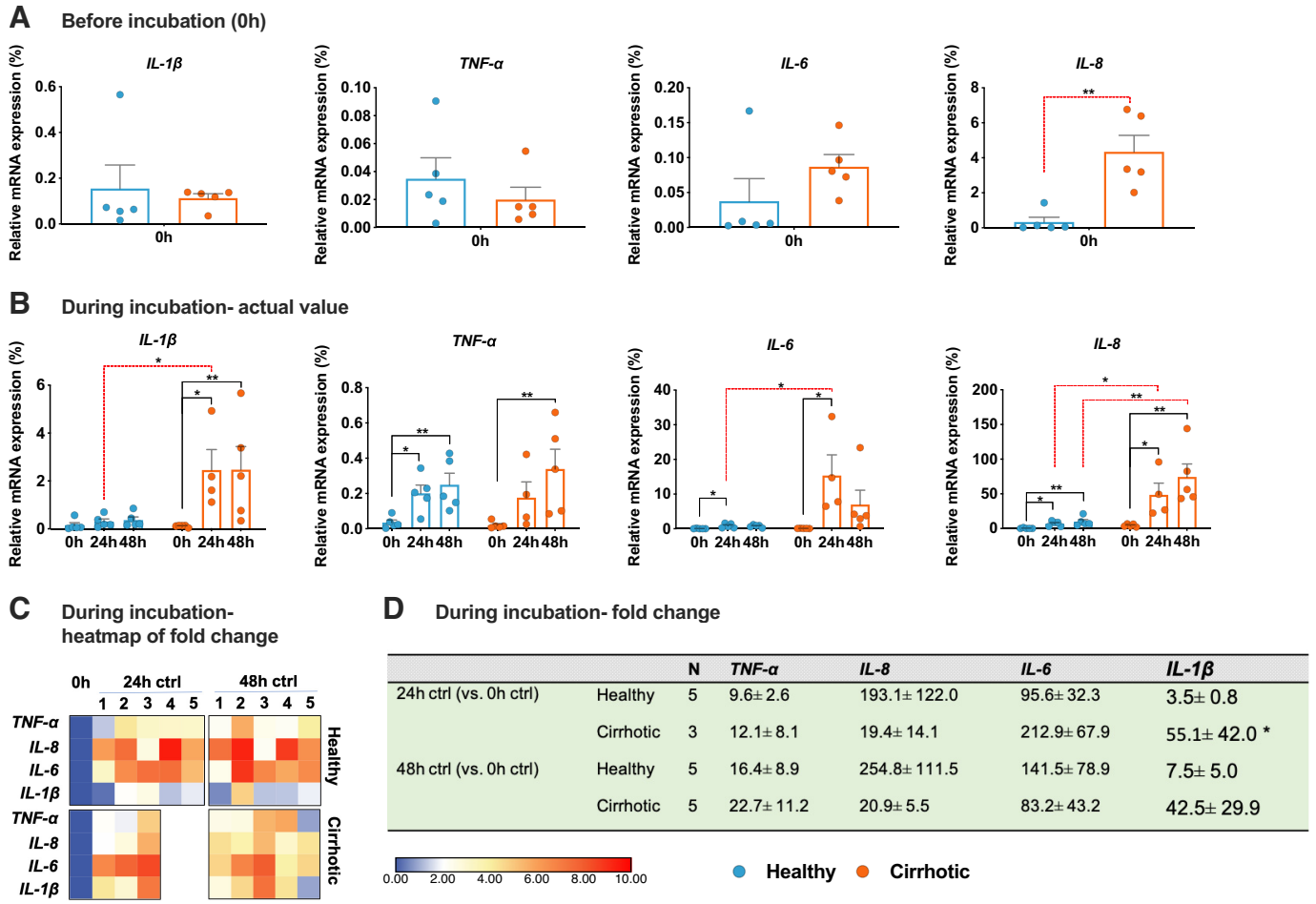
Figure 2. Expression of LPS receptors in human healthy and cirrhotic precision-cut liver slices (PCLSs). Gene expression of the LPS receptors Toll-like receptor 4 (TLR-4), lymphocyte antigen 96 (MD-2), CD180 (RP105), lymphocyte antigen 86 (MD-1), CD14, and Toll-like receptor 2 (TLR-2). *A*: before incubation, relative expression values were expressed as percentage compared with housekeeping gene (100%); *B*: during incubation; *C*: with or without the treatment of LPS. *B*, *C*: fold change of the gene expression compared with the respective control ($2^{-\Delta\Delta Ct}$). *D*: summary table of differently expressed LPS receptors. Data are presented as the means ± SE; healthy: $n = 5$, cirrhotic: $n = 3$–5. $^{*}P < 0.05$ and $^{**}P < 0.01$. LPS, lipopolysaccharide.

DISCUSSION

An altered immune response of cirrhotic liver compared with the healthy livers is reported to contribute to the pathology of ACLF (3). This alteration has never been assessed at a tissue level in the human liver. In this study, we prepared human precision-cut liver slices from cirrhotic and healthy livers to study the onset of inflammation during 48 h of culture with or without LPS challenge. We show for the first time at the tissue level of human livers that, cirrhotic versus healthy liver exhibits distinct immune responses toward incubation and LPS challenge, which might provide valuable information for understanding the pathology as well as for developing new treatment strategies of ACLF.

Our pervious data showed that LPS ranging from 0 to 100 μg/mL produced NOx in a concentration-dependent way. To assess the different phenomena of LPS stimulation, the highest concentration of LPS (100 μg/mL) was used in all initial experiments (19). 100 μg/mL LPS produced 256 nmol NOx at 24 h (assuming that 1 g of liver contains 100 million hepatocytes); the liver slices stimulated with LPS produce an adequate amount of cytokines, which resulted in a similar induction of iNOS as was found in hepatocytes stimulated with LPS and a cytokine mix. Therefore, this preparation seems to be an appropriate in vitro liver system that can mimic the cytokine release of the liver in vivo (19).

The mean LPS activity in serum was 63.0 ± 37.4 pg/mL in incident advanced liver disease (hospitalization, cancer, or death related to liver disease) (Ref. 22). In a case of acute-on-chronic hepatitis B liver failure, the peak phase LPS level is 0.09 EU/mL (23). Plasma LPS levels in patients with sepsis could reach as high as 5.1 ng/mL, with a median value of 300 pg/mL, 25%–75% interquartile range (110–726 pg/mL), and the means (±SD) value was 581 ± 49 pg/mL (24). Sepsis is a trigger for ACLF development, so in ACLF, the LPS concentration could reach as high as nanogram level in plasma (25). In a recent study, a primary cell culture model from ACLF, the researchers used 100 ng/mL to stimulate the immune cells (26). In addition, the LPS concentrations reported in the literature regarding patients with ACLF are from peripheral circulation (plasma), which might be lower than the actual concentration of LPS entering the liver via a portal vein in real-life ACLF (27). The LPS concentration used in this study would be simplification and modeling of this PAMP challenge. Thus, to assess the full phenomena of LPS stimulation, 5 μg/mL was used.

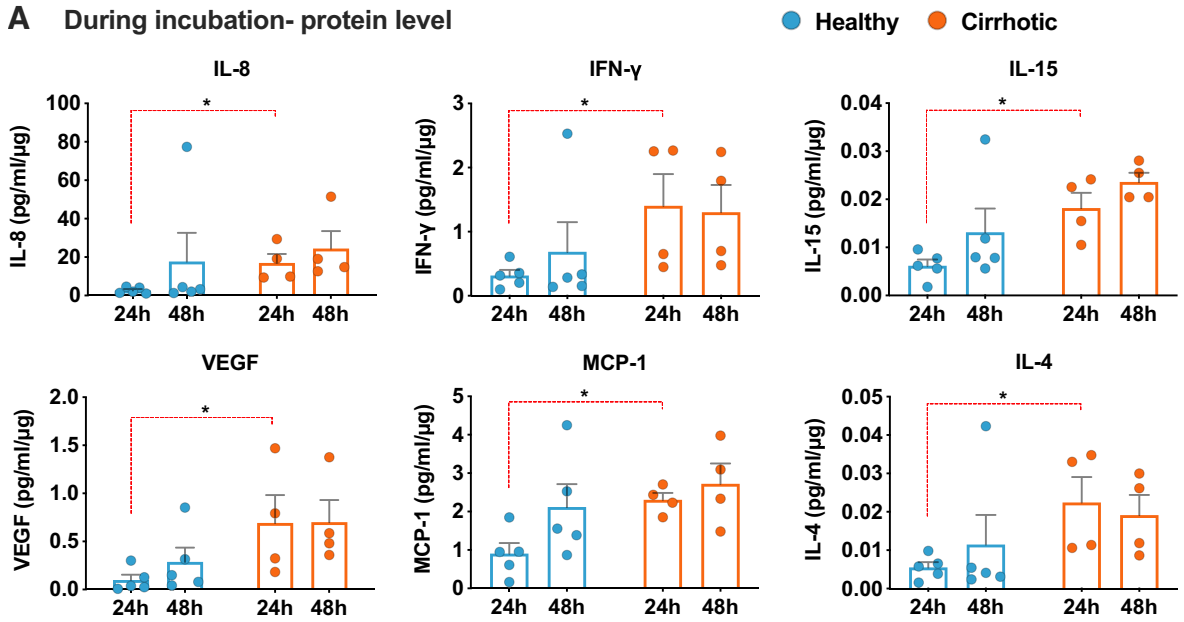


		N	TNF-α	IL-8	IL-6	IL-1β
24h ctrl (vs. 0h ctrl)	Healthy	5	9.6± 2.6	193.1± 122.0	95.6± 32.3	3.5± 0.8
	Cirrhotic	3	12.1± 8.1	19.4± 14.1	212.9± 67.9	55.1± 42.0 *
48h ctrl (vs. 0h ctrl)	Healthy	5	16.4± 8.9	254.8± 111.5	141.5± 78.9	7.5± 5.0
	Cirrhotic	5	22.7± 11.2	20.9± 5.5	83.2± 43.2	42.5± 29.9

Figure 3. Gene expression of proinflammatory markers in the human PCLSs before and during incubation. Gene expression before incubation (*A*) and during incubation (*B*) was detected by qRT-PCR. Expression of proinflammatory genes was expressed as fold change (*C*, *D*). *C*: the gene expression at 0 h was set as 1; fold change of gene expression at 24 h or 48 h was calculated as $2^{-\Delta\Delta Ct}$ and adjusted with Log 2 for illustration. *D*: incubation induced $2^{-\Delta\Delta Ct}$ fold change of gene expression when compared with the 0 h control; data are expressed as means ± SE; *$P < 0.05$ indicates significantly higher in the cirrhotic than healthy PCLSs; others are not significantly different. Healthy: $n = 5$, cirrhotic: $n = 3$–5. IL, interleukin; PCLSs, precision-cut liver slices; TNF-α, tumor necrosis factor-α.

The expression of some of the LPS (co)receptors at baseline, during incubation, and with LPS challenge differed between cirrhotic versus healthy PCLSs. In detail, *TLR-4* and *MD-2* were not differently expressed at baseline, similarly upregulated during incubation and downregulated by LPS in healthy and cirrhotic PCLSs. The innate immune system is activated by LPS through TLR-4 and its coreceptors (28). TLR-4 is associated with secreted MD-2 proteins that recognize LPS to dimerize the TLR-4/MD-2 complexes, initiating the cytoplasmic signaling (29). In line with our data, a previous study showed that LPS downregulates *tlr-4* mRNA expression transiently and severely decreased the surface expression of Tlr-4 protein in the mouse macrophage cell line (RAW264.7) within 24 h, which was in parallel with reduced Il-6 and Il-12 secretion (30). In addition, reduced *TLR-4* and *MD-2* mRNA expression was associated with low responsiveness to LPS in human intestinal epithelial cells (31). In our study, LPS downregulated the *TLR-4* (in both healthy and cirrhotic PCLSs) and *MD-2* (in cirrhotic PCLS) mRNA, showing that both types of livers have the capacity to control the LPS response through regulation of the TLR-4/MD-2 complex.

In healthy PCLSs, *TLR-2* was markedly induced by LPS, whereas in cirrhotic PCLSs, *TLR-2* was expressed, but not influenced by LPS challenge. TLR-2 is the major receptor for lipopeptides and peptidoglycan (32). However, the exact role of TLR-2 in sensing LPS is not fully understood, the current hypothesis is that TLR-2 might act as a signal transductor of TLR-4 signaling or as an independent LPS receptor (32). In an in vitro study using hepatocytes in monoculture, LPS alone was not enough to induce TLR-2 transcription, but IL-1β and TNF-α promoted TLR-2 transcription (33). In our study, LPS stimulated the production of IL-1β and TNF-α, which might explain the upregulation of *TLR-2* observed in PCLSs following LPS treatment. In addition, Vodovotz et al. (33) in their study hypothesized that multiple cell types (e.g., Kupffer cells and hepatocytes) are involved in the mechanism of TLR-2 transcription as a response to LPS. The multicellular nature of the PCLS model lends further credence to the theory that TLR-2 regulation is orchestrated by different cell types. A study in primary human alveolar macrophages showed that LPS treatment for 24 h elevated both mRNA and protein of TLR-2; they proved that full-length TLR-2



B Summary table

	In cirrhotic	In healthy
Higher during incubation	IL-8, IFN-γ, IL-15, VEGF, MCP-1, IL-4	None

Figure 4. Differently expressed cytokines in the culture medium of the healthy or cirrhotic PCLSs. *A*: protein level of the cytokines during incubation; *B*: summary table of differently expressed cytokines during incubation. Bio-Plex multiplex immunoassay of human PCLSs supernatant during incubation of 0–24 h and 24–48 h. Healthy: $n = 5$, cirrhotic: $n = 4$. *$P < 0.05$. PCLSs, precision-cut liver slices.

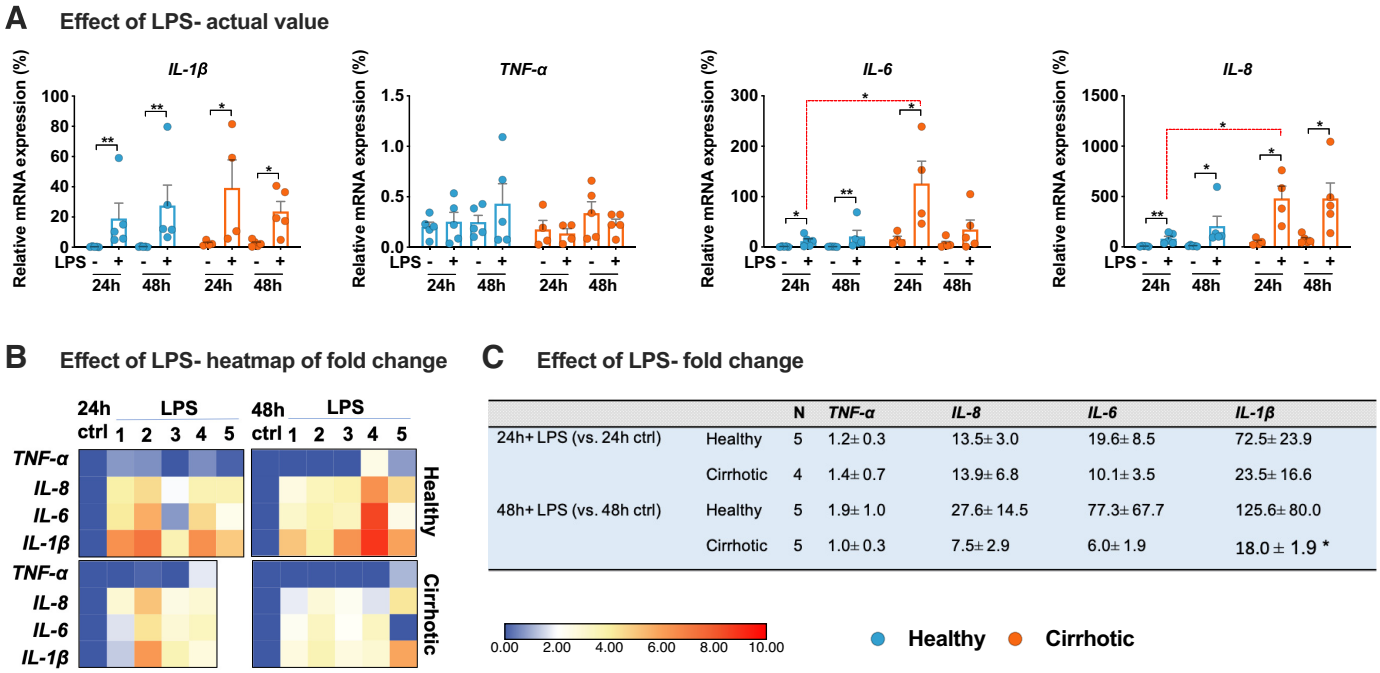
containing extracellular vesicles released by cells under anti-inflammatory conditions has the property of a decoy receptor, possibly contributing to an anti-inflammatory process (34). In summary, the failure of inducing TLR-2 by LPS in the cirrhotic PCLSs might indicate less regulation of inflammation through TLR-2 compared with healthy PCLSs.

Moreover, cirrhotic PCLSs expressed more *RP105*, *MD-1*, *IRAK-M*, and *A20* (Supplemental Fig. S5) than healthy PCLSs at baseline. RP105 and MD-1 are homologs of the major LPS receptors TLR-4 and MD-2, respectively. RP105 and TLR-4 share 22 leucine-rich regions in the extracellular domain, whereas RP105 only has 11 amino acids in the intracellular part and lacks a toll-interleukin receptor domain that initiates the TLR signaling pathway (29). MD-1 is the adaptor molecule for RP105. Together, they were considered to be negative regulators of LPS response in various diseases (35). In a previous study, monocytes from patients with primary biliary cirrhosis were hypersensitive to LPS stimuli, probably due to a downregulated RP105 expression with LPS treatment (36); however, the authors correlated RP105 and increased inflammation, but did not confirm the exact role of RP105. A20 is an inhibitor of NF-κB signaling, which is the downstream of TLR-4 signaling (37). IRAK-M is a negative regulator of TLR-4 signaling (38). CD14, TLR-4, and MD-2 form a multireceptor complex to sense and signal an LPS stimulus (39). Chou et al. showed that CD14 was upregulated transiently during 24 h in liver tissue after LPS stimulation in the bile duct ligated (BDL) rat model. Furthermore, a higher mortality was found in the BDL rat, but CD14 was not changed in the sham-operated liver although endotoxin level was elevated (39). In the current study, only the healthy liver showed the capacity to upregulate the CD14 transiently at 24 h, but not at 48 h or LPS treatment at all time points, which may lead to elevated LPS sensitivity of the healthy than the cirrhotic PCLSs.

Although the overall inflammatory cytokines induced by LPS was pronounced in cirrhotic, the fold changes of them were lower than healthy in our study, which could be due to *1*) the elevated expression of negative regulators of LPS, *RP105*, *IRAK-M*, and *A20* in the cirrhotic PCLSs at baseline; *2*) decreased *RP105* expression in healthy but augmented in the cirrhotic by LPS; and *3*) transient increase of *CD14* expression in healthy at 24 h.

In summary, the receptors, decoy receptors, and negative regulators of TLR-4 are expressed and dynamically regulated during incubation or with the LPS challenge. Most importantly, they are regulated in a different pattern between healthy versus cirrhotic PCLSs. These results suggest that altered LPS receptor expression and regulation in the cirrhotic livers, as compared with healthy livers, contributes at least partially to the altered inflammatory response in the development of ACLF. Future research on the exact mechanism of regulation and involvement of these players in ACLF is encouraged, especially in human samples.

Furthermore, spontaneous increase (upon incubation of the PCLSs) of mRNA of *IL-8*, *IL-6*, *IL-1β*, and protein secretion of IL-8, IFN-γ, IL-15, VEGF, MCP-1, and IL-4 were higher in the



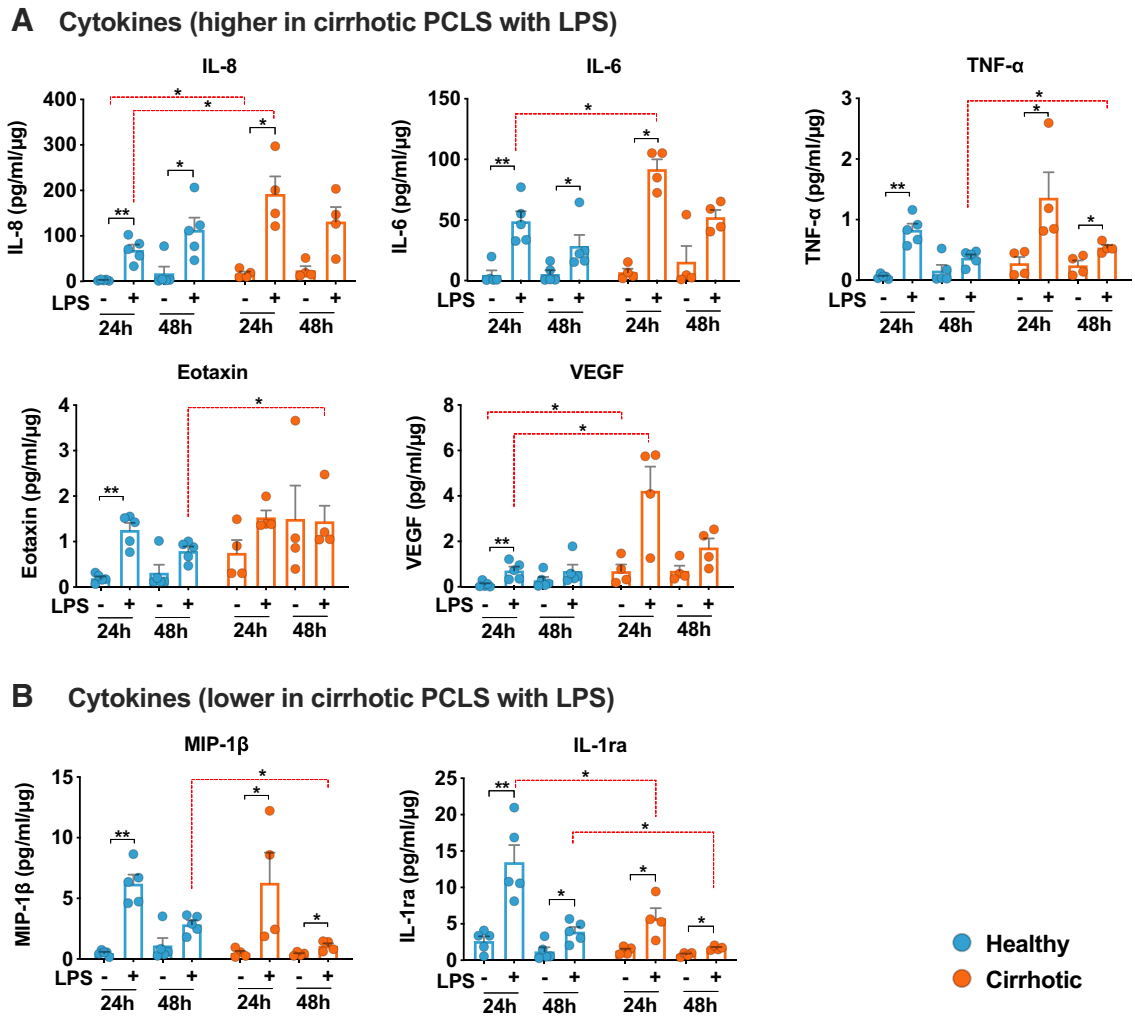
		N	TNF-α	IL-8	IL-6	IL-1β
24h+ LPS (vs. 24h ctrl)	Healthy	5	1.2± 0.3	13.5± 3.0	19.6± 8.5	72.5± 23.9
	Cirrhotic	4	1.4± 0.7	13.9± 6.8	10.1± 3.5	23.5± 16.6
48h+ LPS (vs. 48h ctrl)	Healthy	5	1.9± 1.0	27.6± 14.5	77.3± 67.7	125.6± 80.0
	Cirrhotic	5	1.0± 0.3	7.5± 2.9	6.0± 1.9	18.0 ± 1.9 *

Figure 5. Gene expression of proinflammatory markers in the human PCLSs with LPS challenge. Gene expression of pro-inflammatory markers was detected by qRT-PCR. Relative mRNA expression compared to the house keeping gene (*A*). Expression of proinflammatory genes was expressed as fold change (*B*, *C*). *B*: the gene expression at 24 h or 48 h was set as 1; fold change of gene expression with LPS challenge at 24 h or 48 h was calculated as $2^{-\Delta\Delta Ct}$ and adjusted with Log 2 for illustration. *C*: LPS induced $2^{-\Delta\Delta Ct}$ fold change of gene expression when compared with the respective control at 24 h or 48 h; data are expressed as means ± SE; $^{*}P < 0.05$ and $^{**}P < 0.01$ indicates significantly lower in cirrhotic than healthy PCLSs; others are of no significant difference. Healthy: $n = 5$, cirrhotic: $n = 4$–5.IL, interleukin; LPS, lipopolysaccharide; PCLSs, precision-cut liver slices; TNF-α, tumor necrosis factor-α.

cirrhotic PCLSs, indicating hyperreactive proinflammatory status toward DAMPs stimuli compared with a healthy liver. In line with our observation, monocytes from patients with advanced cirrhosis were spontaneously activated to produce proinflammatory cytokines (15). In addition, serum levels of IL-1β and IL-8 were significantly elevated in patients with primary biliary cirrhosis (16). Moreover, our previous study demonstrated that the preparation and slicing (which involves cold ischemia and mechanical trauma) as well as incubation of PCLSs caused universal transcriptomic and pathway changes to the precision-cut tissue slices (liver, kidney, intestine, and lung) (40). In particular, pathways are involved in IL-6, IL-8, and high-mobility group protein 1 (HMGB1) signaling (40). HMGB1 is secreted by various immune cells or released by injured cells and acts through TLR-2/TLR-4 (41). During cutting and incubation of the slices, HMGB1, representing a DAMP, is released into the culture medium (Supplemental Fig. S6), contributing to the spontaneous inflammatory response in the slices, which might be a resemblance of sterile inflammation caused by necrotic hepatocytes during ACLF. TLR-4 is the receptor for HMGB1, interestingly, the negative regulators of TLR-4, *RP105*, and *MD-1* are increased in the healthy but not cirrhotic PCLSs during incubation, which might be suggesting a better negative regulation of HMGB1 signal transduction in the healthy liver (42). However, the exact role of RP105 and MD-1 in controlling spontaneous inflammatory response in PCLSs and their role in ACLF development needs further examination.

As part of our main interest in elucidating the altered immune response of the cirrhotic liver, we studied the response of cirrhotic PCLSs toward LPS, one of the core proinflammatory agents during ACLF. It was demonstrated previously that repeated LPS exposure causes tolerance to a second LPS stimuli (43). The cirrhotic liver will encounter LPS repeatedly via the gut-liver axis (44). In our study, we showed that cirrhotic livers responded to LPS actively. However, the lower fold change of LPS-induced cytokine level in cirrhotic compared with the healthy as well as higher baseline RP105, MD1, IRAK-M, and A20 in cirrhotic are suggestive of a baseline tolerant phenotype. LPS induced rigorous inflammatory response both in healthy and cirrhotic PCLSs, as 27 of the cytokines tested, 17 were upregulated both in healthy and cirrhotic PCLSs, five were induced only in the healthy (IL-12 p70, basic-FGF, eotaxin, MCP-1, and VEGF) and two were only in the cirrhotic PCLSs (IL-7 and IL-13). IL-5 and IP-10 were not regulated by LPS, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) was not expressed in the PCLSs (Fig. 6, Supplemental Fig. S1).

Cirrhotic PCLSs exhibited an excessive inflammatory response toward LPS stimuli than healthy PCLSs, and produced more proinflammatory cytokines (IL-8, IL-6, TNF-α, eotaxin, and VEGF). In line with our observation, a previous study revealed that monocytes from patients with advanced cirrhosis were hyperresponsive to LPS (15). In addition, peripheral blood mononuclear cells (PBMCs) and human intrahepatic biliary epithelial cells (HIBECs) from primary biliary cirrhosis expressed higher TNF-α, IL-1β, IL-6, and IL-8 than healthy HIBECs after LPS (16). One reason could be that the diseased liver contains more activated myofibroblasts, which possibly produce more cytokines upon LPS stimulus (45). Another reason could be that IL-1ra, an anti-inflammatory protein that blocks IL-1α and inflammation (IL-1β, TNF-α, and



C Summary table

	In cirrhotic	In healthy
Higher with LPS	IL-8, IL-6, TNF-α, Eotaxin, VEGF	MIP-1β, IL-1ra

Figure 6. Bio-Plex multiplex immunoassay of human PCLSs supernatant with LPS challenge. Differently expressed cytokines in the culture medium of the healthy or cirrhotic PCLSs after LPS treatment for 0–24 h and 24–48 h. Cytokines expressed higher (*A*) or lower (*B*) in cirrhotic PCLS with LPS treatment compared with the healthy. *C*: summary table of cytokines differently expressed with LPS treatment in cirrhotiic versus healthy PCLSs. Healthy: $n = 5$, cirrhotic: $n = 4$. *$P < 0.05$; **$P < 0.01$. LPS, lipopolysaccharide; PCLSs, precision-cut liver slices.

MCP-1), was less secreted by cirrhotic PCLSs than healthy PCLSs, leading to an imbalanced inflammation (46, 47). In addition, IL-1ra can initiate blocking of IL-1α and IL-1β, which are capable of inducing IL-8 production, which might explain the lower IL-8 gene expression and secretion in healthy PCLSs than in cirrhotic (48). However, the expression of well-known anti-inflammatory cytokine IL-10 did not differ between the healthy and cirrhotic PCLSs in our study (Supplemental Figs. S4 and S5). Future studies are needed to clarify the role of IL-10 in the context of LPS-induced ACLF.

Furthermore, IL-8 was higher at baseline (gene expression), during incubation (both gene expression and protein) and with LPS (both gene expression and protein) in cirrhotic than in healthy PCLSs. Our finding is in line with a previous report that serum level of IL-8 was significantly elevated in patients with chronic liver disease and this increment was associated with neutrophil or macrophage infiltration to promote liver inflammation (49). These findings show that the cirrhotic liver has a unique basal profile to direct the different inflammatory responses during incubation and with LPS challenge, in which Kupffer cell activation could probably play a role (50).

In addition, we found that cirrhotic PCLSs secreted more monocyte chemotactic protein-1 (MCP-1), which is responsible for monocyte recruitment and closely related to the severity of liver cirrhosis, at 24 h compared with healthy PCLSs

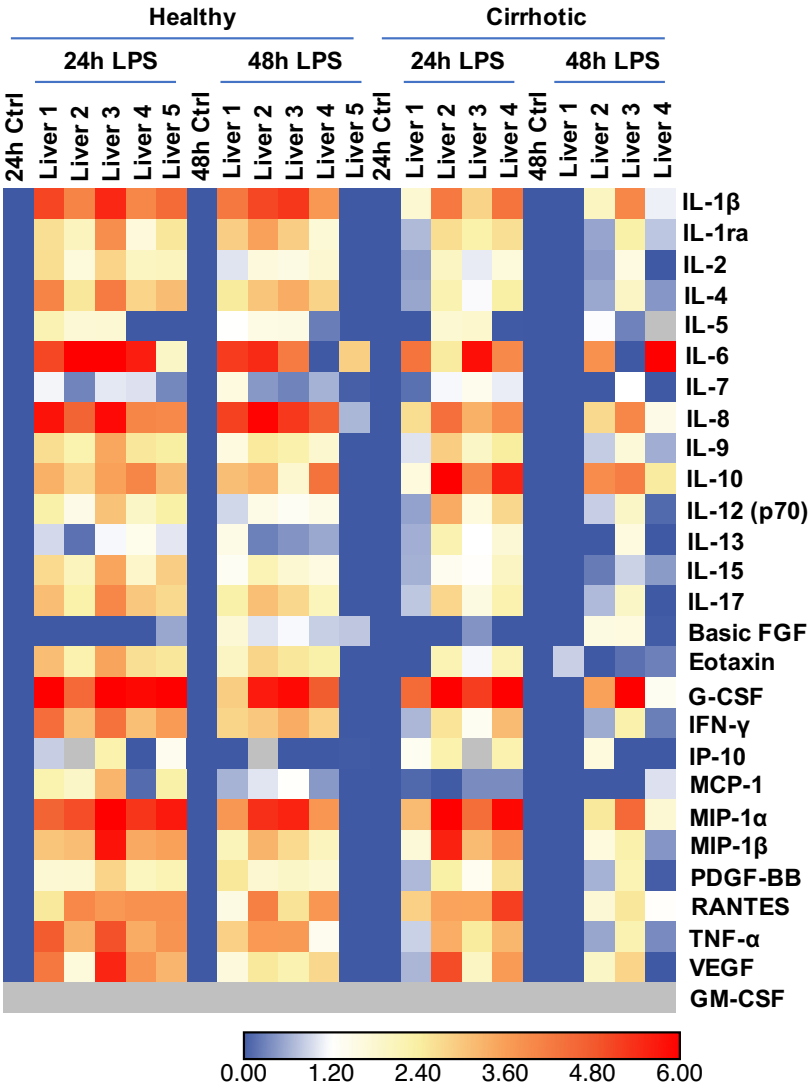


Figure 7. Relative expression of cytokines compared to the respective time control. Cytokines in the culture medium of the healthy or cirrhotic PCLSs after incubation of 0–24 h and 24–48 h with or without LPS treatment were tested with Bio-Plex multiplex immunoassay and the relative expression was calculated as: 24 h LPS/24h control (ctrl) and 48 h LPS/48h ctrl; 24 h ctrl and 48 h ctrl were set as 1. The calculated relative expression was adjusted with Log 2 for heatmap illustration. Healthy: $n = 5$, cirrhotic: $n = 4$. FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN: interferon; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein-1; MIP-1, macrophage inflammatory protein-1; PCLSs, precision-cut liver slices; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor.

(51), and LPS did not induce the release of MCP-1. In contrast, it is upregulated by LPS in the healthy, suggesting a saturated MCP-1 production of the cirrhotic liver before LPS challenge.

Moreover, in the current study, healthy PCLSs produced more macrophage inflammatory protein-1 (MIP-1β), which is a chemoattractant for leukocytes to participate in inflammation, is capable of recruiting regulatory T-cell (CD4 + CD25 +) population, to maintain the normal initiation of T cell and humoral responses and inhibit autoimmunity (52, 53). This finding might indicate a superior ability in leucocyte regulation of healthy PCLSs. Nevertheless, further research on the role of MIP-1β in the cirrhotic liver or patients with ACLF is encouraged. Taken together, several mediators of the inflammatory response in the cirrhotic liver indeed differ from healthy liver, at baseline, during incubation, and upon LPS challenge. These findings provide a better understanding of the imbalanced inflammatory response in cirrhotic livers and might guide the basic research and development of therapeutic approaches for combatting the excessive inflammation during the onset of ACLF.

Conclusions

In summary, our study demonstrates that the expression and regulatory pattern of LPS receptors, spontaneous inflammation, and the response to LPS are different between cirrhotic versus healthy human liver tissue. Cirrhotic PCLSs released markedly more pro-inflammatory cytokines but lower anti-inflammatory cytokines to LPS challenge compared with healthy PCLSs, leading to dysregulated inflammatory response. These events could possibly resemble the inflammatory response of the liver in ACLF. Using human PCLSs for better understanding the pathogenesis of the disease may guide the novel therapeutic approaches to mitigate the excessive inflammatory response during the onset of ACLF, which might result in less additional damage to the already damaged cirrhotic liver and hopefully a better outcome in patients with ACLF.

SUPPLEMENTAL DATA

Supplemental Tables S1–S5; Supplemental Figs. S1–S6: https://doi.org/10.6084/m9.figshare.19695304.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.S., T.L., and P.O. conceived and designed research; S.S., M.L., T.L., M.B., D.O., and A.R.G. performed experiments; S.S., T.L., M.B., and L.B. analyzed data; S.S., T.L., M.B., K.P.d.J., and L.B. interpreted results of experiments; S.S., M.L., T.L., and A.R.G. prepared figures; S.S. and T.L. drafted manuscript; T.L., K.P.d.J., L.B., and P.O. edited and revised manuscript; K.P.d.J., L.B., and P.O. approved final version of manuscript.

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