Journal Pre-proof

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PII: S0168-8278(20)30028-3

DOI: <https://doi.org/10.1016/j.jhep.2020.01.011>

Reference: JHEPAT 7597

To appear in: *Journal of Hepatology*

Received Date: 9 October 2019

Revised Date: 22 December 2019

Accepted Date: 14 January 2020

Please cite this article as: Engelmann C, Sheikh M, Sharma S, Kondo T, Loeffler-Wirth H, Zheng YB, Novelli S, Hall A, Kerbert AJC, Macnaughtan J, Mookerjee R, Habtesion A, Davies N, Ali T, Gupta S, Andreola F, Jalan R, Toll-like receptor 4 is a therapeutic target for prevention and treatment of liver failure, *Journal of Hepatology* (2020), doi: <https://doi.org/10.1016/j.jhep.2020.01.011>.

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Toll-like receptor 4 is a therapeutic target for prevention and treatment of liver failure

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Data and materials availability: Data are available on request from the corresponding author (Prof. Rajiv Jalan).

Key words: ACLF, ALF, TLR4, inflammation, liver injury, lipopolysaccharide, LPS, DAMP, PAMP

Electronic word count: 6706

Number of figures: 4

Number of tables: 0

Competing interests: Rajiv Jalan has research collaborations with Takeda, and Yaqrit, and consults for Yaqrit. Rajiv Jalan is the founder of Yaqrit Limited, which is developing UCL inventions for treatment of patients with cirrhosis. Rajiv Jalan is an inventor of ornithine phenylacetate, which was licensed by UCL to Mallinckrodt. He is also the inventor of Yaq-001, DIALIVE and Yaq-005, the patents for which have been licensed by his University into a UCL spinout company, Yaqrit Ltd. Cornelius Engelmann has on-going research collaboration with Sequana Medical, Merz Pharmaceutical and Novartis. He has received speaker fees from Novartis, Gilead and Merz Pharmaceuticals.

Funding: This study was financially supported by Takeda (Pharmaceuticals U.S.A, Deerfield, USA). The German Research Foundation (DFG) funded Cornelius Engelmann (EN 1100/2-1).

Author contributions: RJ, MS, FA, CE, FA, ND - contributed to the conception and design of the study. RJ, ND, FA - provided administrative, study supervision, obtained funding, material support. CE, MS, SS, TN, HLW, YBZ, SN, AH, RM, AH - performed experiments and substantially contributed to the acquisition of data and its analysis. All authors were involved in the interpretation of data. CE drafted the manuscript. All authors revised the manuscript critically for important intellectual content.

Abstract

Background and aims: Toll-like receptor 4 (TLR4) plays an essential role in mediating organ injury in acute liver failure (ALF) and acute-on-chronic liver failure (ACLF). Here we assess whether inhibiting TLR4 signaling can ameliorate liver failure and serve as a potential treatment.

Material and Methods: Circulating TLR4 ligands and hepatic TLR4 expression was measured in plasma samples and liver biopsies from patients with cirrhosis. TAK-242 (TLR4 inhibitor) was tested *in vivo* with 10mg/Kg, i.p. in rodent models of ACLF (bile duct ligation + lipopolysaccharide (LPS); carbontetrachloride + LPS) and ALF (Galactosamine + LPS) and *in vitro* on immortalized human monocytes (THP-1) and hepatocytes (HHL5).. The *in vivo* therapeutic effect was assessed by coma free survival, organ injury and cytokine release and *in vitro* by measuring IL6, IL1b or cell injury (TUNEL), respectively.

Results: In patients with cirrhosis, hepatic TLR4 expression was upregulated and circulating TLR4 ligands were increased ($p<0.001$). ACLF in rodents was associated with a switch from apoptotic cell death in ALF to non-apoptotic forms of cell death. TAK-242 reduced LPS induced cytokine secretion and cell death ($p=0.002$) in hepatocytes and monocytes *in vitro*. In rodent models of ACLF, TAK-242 administration improved coma free survival, reduced the degree of hepatocyte cell death in liver ($p<0.001$) and kidneys ($p=0.048$) and reduced circulating cytokine levels (IL1b $p<0.001$). In a rodent model of ALF TAK-242 prevented organ injury ($p<0.001$) and systemic inflammation (IL1b $p<0.001$).

Conclusion: This study shows that TLR4 signaling is a key factor in the development of both ACLF and ALF and its inhibition improves severity of organ injury and outcome. TAK-242 may be of therapeutic relevance in patients with liver failure.

Lay summary

TLR4 is mediating endotoxin induced tissue injury in liver failure and cirrhosis is associated with a TLR4 related sensitization to endotoxins.

TLR4 signaling inhibition by TAK-242 ameliorates organ injury and systemic inflammation in rodent models of acute and acute-on-chronic liver failure.

Graphical abstract

Highlights:

Cirrhosis is associated with toll-like receptor 4 (TLR4) related organ sensitization to endotoxins and a switch from apoptotic cell death in acute liver failure to necroptotic cell death in acute-on-chronic liver failure.

TLR4 signaling inhibition by TAK-242 reduces immune cell response (THP1) and hepatocyte injury (HHL5) in response to LPS *in vitro*.

In rodent models of acute and acute-on-chronic liver failure TAK-242 mitigated LPS driven systemic inflammation and organ injury.

Introduction

Acute-on-chronic liver failure (ACLF) is a catastrophic syndrome that occurs in patients with cirrhosis who decompensate acutely usually due to a precipitating event and rapidly progress to multiorgan failure (1). It occurs in about 30% of hospitalized cirrhotic patients and is associated with a 28-day mortality varying between 20% in ACLF grade 1 and 70% in ACLF grade 3, thus being significantly higher than 5% in ‘mere’ acute decompensated liver cirrhosis (1, 2). Systemic inflammation possibly induced by high levels of circulating pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) are the pathophysiological hallmark of this syndrome (3, 4). Emerging lines of investigations suggest that cirrhosis is associated with processes sensitizing organs to precipitating events, such as bacterial infections, which are relatively straightforward to manage in healthy individuals with very low mortality rates, but might lead to multi-organ failure and high risk of short-term mortality in patients with cirrhosis. These clinical observations are in concordance with previous findings in rodent models of chronic liver injury, in which animals develop features of multiorgan failure in association with high mortality rates following exposure to PAMPs such as lipopolysaccharide (LPS) at doses that are highly well-tolerated by healthy animals (5, 6).

Acute liver failure (ALF) is distinct from ACLF as it develops in patients with healthy liver after exposure to hepatotoxins, notably drugs, viral hepatitis or in autoimmune hepatitis (7). Although

the first short period of the disease is pathomechanistically most often linked to a direct toxin – hepatocyte interaction and secondary inflammatory reactions as a consequence of cell death and immune activation lead to extra-hepatic organ failures (8). As a result of liver injury, patients often present with endotoxemia (9) and increased levels of plasma inflammatory cytokines such as IL1 β , IL6 and TNF α (10). Importantly, inhibition of cell-death has been shown to prevent progression of ALF (11).

Therefore, in both ACLF and ALF, inflammation plays a key role in the current pathomechanistic paradigm of the development of organ failures and disease course. Toll-like receptor 4 (TLR4) is a pattern recognition receptor. Its major ligands are the PAMP lipopolysaccharides (LPS), gram-negative endotoxins (12) and DAMPs, such as cell death products (cleaved nucleosomes, histones, HMGB1) (13). This plasma membrane receptor is expressed on a multitude of non-parenchymal and parenchymal cells, including hepatocytes and hepatic stellate cells (14, 15). Upon ligand-binding, the receptor dimerises and triggers the recruitment of adaptor molecules, including TIR domain-containing adaptor protein (TIRAP)-MyD88 and TRIF-related adaptor molecule (TRAM)-TRIF, leading to the formation of an intra-cellular signaling complex (16). The MyD88-dependent signaling results in activation of NF- κ B whereas the TRIF-dependent pathway regulates interferon regulatory factor leading to cytokine and interferon production (16). In a rat model of ACLF (bile duct ligation (BDL) followed by LPS injection), renal TLR4 was found to be up-regulated and mediated endotoxin-related kidney injury. Reducing TLR4 signaling by gut decontamination prevented the development of kidney injury (17). The effect of TLR4 genetic impairment and inhibition was also studied in a mouse model of acetaminophen-induced acute liver failure. Both interventions on the LPS-TLR4

pathways reduced the amount of cell death, improved renal function and reduced the coma-free survival rate (18). Both studies show that the LPS-TLR4 axis is substantially involved in inflammatory processes leading to tissue injury and that the inhibition of this signaling pathway prevents disease progression.

TAK-242, a small molecule TLR4 antagonist, selectively disrupts TLR4 signaling by direct binding to the intracellular TIR domain resulting in impaired recruitment of both TIR domain-containing adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM) (16) thereby causing a reduced production of cytokines, TNF α , IL6 and nitric oxide (NO) (16). TAK-242 also abrogates collagen synthesis and myofibroblast differentiation in fibroblasts (19) and prevents liver fibrosis in murine and rodent models (20). Preliminary studies in BDL rat models reduced the severity of organ injury (21, 22). Therefore, TAK-242 may be a potential therapy for patients with ALF and ACLF. The aims of this study were to evaluate the relevance of the TLR4 pathway in both ACLF and ALF and determine whether TLR4 signaling inhibition with TAK-242 could serve as a potential therapy for these groups of patients.

Materials and Methods

Study design:

This study aimed at characterizing the role of the LPS-TLR4 pathway in mediating organ injury and to evaluate the efficacy of a TLR4-signaling inhibition to mitigate liver failure. Human samples were retrieved after obtaining ethical approval during clinical routine interventions. Murine *in vivo* models for ALF and ACLF were used to characterize the LPS-TLR4 pathway and

for TAK-242 testing. The sample size for the BDL experiment was calculated based on the results provided by Shao Z et al (23) showing an ALT reduction of about 70%. We hypothesized conservatively an effect size of 30% in our model with alpha error of 0.05 and power of 80% calculating a samples size of 7 animals per group. All animals were included in the analysis unless specified in the figure legend. Animals were randomly assigned to experimental interventions but no blinding was applied. The efficacy of TAK-242 to inhibit TLR4 signaling was confirmed *in vitro* in human monocytes (THP-1), human hepatocytes (HHL5) and HEK-TLR4 reporter cells.

Human cohort

Human liver biopsies from healthy controls (n=2) and patients with alcoholic hepatitis (n=12) were obtained from the histology department of the Royal Free Hospital London (UCL Biobank; Ethical approval: REC number 07/Q0501/50) and from patients with chronic hepatitis B induced ACLF (n=7) from the Third Affiliated Hospital of Sub Yat-Sen University in Guangzhou, China, (Human Ethics Committee of the Third Affiliated Hospital, approval number ZSSYME(2016)2-72) during clinically indicated liver biopsy or liver transplantation.

Human plasma samples from patients consecutively admitted with acute decompensated liver cirrhosis to the Royal Free Hospital London and suitable for this study were collected prospectively (Ethical approval: REC number 08/H0714/8) and individual samples were used (unselected) to measure circulating LPS and nucleosome levels as well as HEK-Blue hTLR4 cell response, each tested in duplicates.

All the samples were collected with informed consent from the patients and according to the principles of good clinical practice and the Declaration of Helsinki.

Animal models

Bile duct ligation model

The rat model used to mimic ACLF in this study was published and described by Harry D et al. (6). Male Sprague–Dawley rats (weights 260 +/-20g, age 8-10 weeks) were studied 4-weeks after either sham-operation or bile duct ligation (BDL). Lipopolysaccharides (LPS) derived from *Klebsiella pneumonia* (0.025 mg/kg) (Sigma, UK) was injected intraperitoneally (i.p.) as a second hit to induce organ injury. TAK-242 (Takeda, JP – Lot number M342-017) treatment was prepared as follows. Briefly, TAK-242 was redissolved in N-2 methyl-pyrrolidone (NMP) to make a 200mg/ml stock solution which was then further diluted to an intermediate concentration of 40mg/ml in NMP. The 40mg/ml solution was finally diluted to a working concentration of 1mg/ml in a solution of 30%(2-hydroxypropyl) β -cyclodextrin (Sigma, Cat No H107)/0.5% Citric acid. The vehicle control solution was prepared by adding NMP to 30%(2-hydroxypropyl) β -cyclodextrin/0.5% Citric acid (1:40 ratio). TAK-242, 10mg/kg i.p, was used either prophylactically (3 hours prior to LPS injection) or therapeutically (45 min post LPS). After treatment(s) administration, rats were kept in a temperature controlled environment and allowed free access to food and water. After 6 hours (or 3 hours post LPS when TAK-242 administration preceded LPS) rats were put under general anaesthesia (2% isoflurane in oxygen) and systemic haemodynamics were measured, as described below, before sacrificed by exsanguination.

The groups were as follows:

1. Sham (n=9)

2. BDL (n=8)
3. BDL + LPS (n=7)
4. BDL + TAK-242 + LPS (prophylactic approach) (n=7)
5. BDL + LPS + TAK-242 (therapeutic approach) (n=16)

Mean arterial Pressure (MAP) and portal pressure (PP) were measured whilst animals were under anaesthesia by direct cannulation of the right carotid artery and main portal vein, respectively. Measurements were transduced to a Powerlab (4SP) linked to a computer with Chart v5.0.1 software.

Carbon tetrachloride model

In this rodent model, male C57BL/6 mice (weights $30\text{g} \pm 5\text{g}$, age 8-10 weeks) were gavaged with carbon tetrachloride (CCl_4 0.5mg/ml dissolved in olive oil – dose 0.5ml/kg) twice weekly for a total of 6 weeks to induce a chronic liver injury as described previously (5). LPS derived from *Klebsiella pneumonia* (Sigma, UK) (4mg/kg) or vehicle (saline solution) was injected i.p. to mimic ACLF. Treatment with TAK-242 (Takeda Pharmaceuticals U.S.A, Deerfield, USA) 10mg/kg i.p. was initiated 45min after LPS and repeated after 22h. Animals were sacrificed 24 hours after LPS injections.

The groups were as follows:

1. Vehicle (n=10)
2. CCl_4 (n=10)
3. CCl_4 + LPS (n=6)
4. CCl_4 + LPS + TAK-242 (n=4)

Galactosamine model

In order to induce acute liver failure without pre-existing chronic liver injury D-galactosamine (GalN) was injected i.p. in combination with LPS at a dose of 400mg/kg (GalN) and 0.05mg/kg (LPS, Klebisella) diluted in saline solution (0.9% NaCl) in adult male Sprague–Dawley rats (weights 260 +/-20g, age 8-10 weeks). All animals were sacrificed 6 hours after GalN+LPS administration. Treatment with TAK-242 (Takeda Pharmaceuticals U.S.A, Deerfield, USA) 10mg/kg i.p was given 45 min after GalN+LPS injection

The groups were as follows (supplementary figure 1):

1. Vehicle (n=10)
2. GalN (n=5)
3. GalN + LPS (n=10)
4. GalN + LPS + TAK-242 (n=7)

Sampling and Storage

Blood samples were taken from the abdominal aorta or right heart as appropriate. EDTA and/or lithium heparin plasma was centrifuged 2500 rpm for 10 min and stored at -80°C for later analysis. All tissues (liver, brain, kidneys) were snap frozen in liquid nitrogen and stored at -80°C for further analysis. In addition, organs were harvested in formalin (48h) for histological assessment.

Human samples were tested for liver TLR4 expression, circulating nucleosome and LPS levels and HEKBlue-hTLR4 reporter cell stimulation.

The therapeutic effect of TAK-242 was assessed by coma free survival, organ injury and cytokine release. Detailed descriptions of endpoint analyses are provided in the supplementary.

Statistical analysis

All statistical analysis was performed using SPSS 22 software (SPSS Inc., Chicago; IL). Group comparisons for continuous variables were performed by using Man-Whitney U test and for categorical variables by using Chi-Square test. If more than two groups had to be analysed one-sided ANOVA with post-hoc Tukey analyses for multiple comparison was used. A p-value ≤ 0.05 was considered significant. Graphs were prepared with Prism (GraphPad, USA) and figures compiled in Adobe Photoshop (Adobe Systems, USA).

Study approval

All animal experiments were in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 (updated 2012) and a project license (No.: 14378) provided by the UK Home Office.

Results

TLR4 pathway is up-regulated in patients with cirrhosis

As previously discussed, PAMPs and DAMPs, many of which signal through the TLR4 pathway, play a prominent role in mediating inflammation and organ injury in liver failure. In cirrhosis, it is likely that there is organ sensitization to TLR4 ligands such as endotoxins (24). LPS levels are known to be elevated in plasma of patients with cirrhosis as compared to healthy controls (4). We therefore assessed the significance of the LPS-TLR4 pathway in mediating organ injury in liver cirrhosis. Firstly, we analyzed the activity of circulating TLR4 ligands by incubating a HEK-TLR4 reporter cell line with plasma samples from a cohort of 10 healthy controls, 17 patients with compensated cirrhosis and 151 patients with acute decompensated cirrhosis (acute decompensation n=87, ACLF n=64) (fig 1A, table S1, fig S7). Circulating endotoxin and nucleosome concentrations (the most prominent PAMP and DAMP) increased numerically in plasma from patients with acute decompensation but did not reach statistical significance (fig 1B). However, plasma from patients with acute decompensation triggered a significant cell response by increasing the mean fold induction to untreated controls from 1.01 ± 0.44 in healthy controls, over 1.61 ± 0.45 in compensated cirrhosis to 1.77 ± 1.19 in acute decompensated cirrhosis ($p < 0.001$ to healthy controls) (fig 1B). These data suggest that plasma from patients with decompensated cirrhosis has the ability to increasingly transactivate the TLR4-receptor but the response is not exclusively mediated by single molecules but rather by a multitude of circulating TLR4 ligands (fig 1B), especially as there was no correlation between both parameters ($r = -0.067$, $p = 0.631$). There was no significant difference between patients with acute decompensated cirrhosis and ACLF (fig S7).

Thereafter, liver TLR4 protein expression, as a surrogate for TLR-related organ sensitization, was assessed by immunohistochemistry in liver biopsies from patients with cirrhosis (alcoholic hepatitis n=12, chronic hepatitis B n=7) and healthy controls (n=2) (table S3). Hepatic TLR4 expression was significantly greater in both cohorts with cirrhosis (fig 1C) suggesting that the occurrence of cirrhosis sensitizes the liver to TLR4 ligands via TLR4 receptor up-regulation. Therefore, as circulating TLR4 ligands are also increased, it was hypothesized that individuals with cirrhosis are predisposed to TLR4-receptor mediated organ injury.

TAK-242 ameliorates in vitro cell response to LPS in monocytes and hepatocytes

TAK-242 is a selective small molecule TLR4 inhibitor (LPS-induced cytokine in human whole blood (IC_{50} μ M) = 0.87). We therefore tested *in vitro* whether TAK-242 is able to block the LPS-induced response in human immortalized monocytes (THP-1) and hepatocytes (HHL5), both important mediators of organ injury in liver failure (25). TLR4 expression in THP-1 cells has been described before (26). For both THP-1 and HHL-5 TLR4 mRNA expression was confirmed using qPCR (data not shown). For HHL5 cells, this was additionally shown using immunofluorescence staining (fig S1). THP1 monocyte and macrophage (PMA stimulated) response to LPS was assessed by measuring IL6 mRNA expression after 5 hours; IL1b secretion into the cell supernatant in THP1 monocytes after 24 hours; and LPS-induced apoptosis in HHL5 by TUNEL staining and caspase 3/7 enzyme activity (fig 1D). Incubation of THP-1 cells with LPS for 5-hours resulted in IL6 transcription in both THP1 cell subsets, which was reduced by pre-incubation with TAK-242 (fig 1E). The same effect was observed for IL1b in the supernatant of unstimulated THP1 monocytes (fig S8). The apoptotic rate of HHL5 was expressed in percentage of TUNEL positive cells. LPS, at a dose of 100ng/ml, significantly increased the

relative numbers of apoptotic nuclei, which was highly significantly reduced when cells were pre-incubated with TAK-242. Enzyme activity of caspase 3/7, mediators of apoptotic cell death, increased with LPS incubation but was ameliorated when co-incubated with TAK-242 (fig 1F). Therefore, TAK-242 was effective in diminishing the TLR4-mediated response to LPS in hepatocytes and monocytes.

Prevention and treatment of ACLF with TAK-242 in a rodent model

The protective effect of TAK-242 on organ injury was assessed in two distinct animal models of ACLF. To mimic advanced chronic liver injury, Sprague Dawley rats underwent a bile duct ligation operation. Four weeks after surgery, an intraperitoneal injection with LPS served as a second hit leading to extra-hepatic organ failure. Animals were sacrificed at 6-hours after LPS injection or at time of severe coma or death within that period. TAK-242 (10mg/kg i.p.) was injected either 3-hours before LPS injection (prophylactic) or 45 min after (therapeutic). The second model of advanced chronic liver disease in mice was by gavaging with carbon tetrachloride (CCL₄) for 6-weeks and also injected with LPS thereafter. These animals were sacrificed 24 hours after the LPS injection. Two injections of TAK-242 were administered (10 mg/kg i.p.) at 45 minutes and 22 hours after LPS injection (fig 2A and D). The presence and activity of TAK-242 in the blood of the treated animals was assessed *in vitro* by using the HEK-TLR4 reporter cells. Incubation with plasma from BDL+LPS rats induced a HEK-TLR4 cell response equivalent to 0.43 ng/mL LPS, whereas there was no cell response after exposure to plasma from TAK-242-treated animals (0.04 ng/mL LPS equivalent, $p=0.016$) (fig S2A).

Coma-free Survival and Circulation: Within 6-hours after LPS injection, 87% of the BDL animals developed severe coma or died. In the TAK-242 pre-treated animals, coma-free survival was 100% and 53% animals survived when TAK-242 was administered in the treatment mode. Also, the mean arterial pressure was significantly reduced after LPS injection ($p=0.003$) but tended to improve during treatment with TAK-242 (fig 2B).

Liver: Bile duct ligation over 4-weeks induced a chronic liver injury with extended biliary regenerative areas in combination with inflammatory cell infiltrates and apoptosis ($p<0.001$) following LPS injection (fig 2C). ALT levels increased from 46.5 ± 8.8 U/L in BDL to 129.4 ± 33 U/L ($p<0.001$) in BDL+LPS. Liver injury was significantly reduced by TAK-242 in both, prophylactic and therapeutic treatment approaches (fig. 2C). In the same groups the TUNEL positive areas in liver tissue decreased from $13.3 \pm 1.8\%$ to $7.7 \pm 1.2\%$ ($p<0.001$) and $7.4 \pm 2.4\%$ ($p<0.001$), respectively and ALT levels were significantly reduced to 66.2 ± 9.4 U/L ($p<0.001$) and 86.1 ± 22.8 U/L ($p=0.002$). In order to further characterize the type of LPS-induced cell death, we measured caspase 3/7 and 8 enzyme activities in liver lysates and RIPK3 levels in plasma of rats. Chronic liver injury induced by BDL significantly increased caspase 3/7 activity ($p<0.001$) and numerically caspase 8 activity and was associated with low RIPK3 levels. LPS injection in BDL animals led to a switch from apoptotic (caspase 3/7-mediated) cell death in BDL animals to predominantly necroptotic (RIPK3-mediated) cell death in BDL+LPS animals, characterized by reduced caspase 3/7 ($p=0.003$) and caspase 8 activities and high plasma levels of RIPK3 ($p<0.001$). TAK-242 significantly abrogated LPS-induced necroptosis. RIPK 3 levels were reduced (prophylactically $p=0.099$, therapeutically $p=0.035$) with remaining low levels of caspase 3/7 and 8 activities (fig S4).

Kidneys and Brain: LPS induced cell death in the kidneys (TUNEL) ($p=0.003$) and led to deterioration in renal function (plasma creatinine) ($p<0.001$). Both effects were significantly improved by TAK-242 (fig 2C). There was a tendency towards increasing relative brain water content after LPS injection, which was improved with TAK-242 ($p=0.03$).

Circulating cytokines: The development of multi-organ injury after LPS injection was accompanied by significant increased plasma levels of IL1 β ($p<0.001$), TNF α ($p<0.001$), IL10 ($p<0.001$) and IL6 ($p<0.001$), which were significantly attenuated by TAK-242, both in the prophylactic and treatment modes (fig S3A).

Validation of the effect of TAK-242 in a distinct rodent model of ACLF: To validate these results, TAK-242 (10 μ g/kg i.p.) was tested in CCL4+LPS treated mice as a second rodent model of ACLF, which is characterized by lower mortality as compared to the previously describe BDL+LPS rat model and therefore allows a longer follow up period after LPS injection. All mice survived spontaneously until being sacrificed. Animals treated with CCl₄ for 6 weeks developed bridging fibrosis in the liver (fig 2E). LPS injection induced apoptotic and necrotic areas in the liver and kidneys (TUNEL). TAK-242 treatment over 24 hours diminished LPS related cell death in both organs (fig 2E+F). Also, ALT levels increased with LPS but improved with TAK-242 (fig 2E). CCL5, ICAM-1 and NGAL levels in liver lysates, as markers for inflammation, organ injury and regeneration, respectively, increased with LPS injection but were markedly reduced after TAK242 treatment (fig S3B).

Galactosamine/LPS induced acute liver injury can be treated with TAK-242

As shown by Shah et al., TLR4 also plays a role in mediating tissue injury in ALF (18). We therefore tested the effect of TAK-242 in an ALF rat model with Galactosamine (GalN) + LPS (fig 3A). Whilst hepatocytes in healthy livers are relatively protected against endotoxin-related effects, GalN sensitizes hepatocytes to LPS-induced NF- κ B mediated cell injury (27).

Liver: GalN+LPS triggered a significant liver injury with increasing ALT levels ($p < 0.001$) (fig. 3B) and TUNEL positive areas ($p < 0.001$) (fig 3B). Treatment with TAK-242 significantly reduced the GalN+LPS induced acute liver injury. Whilst plasma levels of ALT (275.6 ± 176.9 U/L, $p = 0.66$) showed a trend towards improvement (fig. 3A), TAK-242 markedly reduced areas of cell death (TUNEL) in the liver ($0.28 \pm 0.14\%$, $p < 0.001$) (fig 3B). Cell death could be significantly attributed to apoptosis as caspase 3/7 enzyme activities were significantly increased ($p = 0.015$) after GalN+LPS without relevant increase of RIPK3 plasma levels (fig S4). TAK-242 treatment resulted in decreased caspase 3/7 enzyme activities ($p < 0.05$) (fig S4).

Circulating cytokines: Circulating IL1 β cytokine level increased from 0.1 ± 0 pg/mL in controls to 107.9 ± 69.7 pg/mL ($p < 0.001$), which was dramatically improved towards normality with TAK-242 (0.1 ± 0 pg/mL, $p < 0.001$). All other cytokines (IL6, TNF α , IL10) showed the same trend, however, without reaching significance (fig S3A).

Kidneys and Brain: Other organs were less affected by GalN+LPS but tended to improve with TAK-242. TUNEL positive areas in kidney were reduced from $6.4 \pm 4.2\%$ to $1.6 \pm 0.97\%$,

$p=0.051$, whilst creatinine plasma levels remained unchanged (fig. 3B). The relative brain water content remained unchanged after GalN+LPS, but this was significantly reduced with TAK-242 ($p<0.001$) (fig S5).

Toll-like receptor 4 pathway modulation

In order to better define the mechanism of the effect of TAK-242 we characterized the TLR4 signaling pathway activation after induction of liver injury and during treatment with TAK-242.

TLR4 mRNA and protein expression is increased in cirrhosis: Immunohistochemical staining of liver tissue showed that chronic liver injury, either induced by BDL or CCL4, lead to TLR4 up-regulation ($p<0.001$, $p=0.008$) (fig 4A), also confirmed at mRNA expression level (BDL 5.3 fold upregulation, BDL-LPS 7.7 fold up-regulation) (fig S6); these findings are in accordance with the results obtained from human liver tissue (fig 1). In ALF induced by GalN, hepatic TLR4 receptor expression remained unchanged at the protein ($p=0.999$) (fig 4A) and mRNA level (fig S6).

TLR4 signaling in ACLF and ALF models: TLR4 pathway gene expression showed a markedly different pattern between both models (fig. 4C). In GalN induced ALF, mRNA expression of effector genes such as TNF α and IL6 increased 31.8 fold (CI 34.5; 29.3) and 318 fold (CI 332.5; 304.9), respectively, and were down-regulated significantly by TAK-242 (fig 4D). In the ACLF model, hepatic mRNA cytokine levels in response to LPS were generally lower compared to the ALF model (fig 4D). We also measured mRNA levels for TLR4 regulator genes MyD88 and

TRAF6. In general, TLR4 signals through a cascade of regulatory proteins with enzymatic activity ending in a transcriptional modulation of cytokine effector molecules. The mRNA expression of cytokines such as TNF α and IL6 depicts direct pathway activation whereas mRNA levels of regulatory genes resemble a secondary regulatory feedback to the actual pathway activity. As a consequence, inhibition of TAK-242-related pathway reduced cytokine mRNA expression in both models (fig 4D) whilst the mRNA pattern of regulatory genes was distinct. MyD88 and TRAF6 were down-regulated more than 2-fold (fig. 4D) after GalN+LPS and remained unchanged during treatment. In BDL+LPS animals, chronic liver injury induced by BDL led to a down-regulation of MyD88 and TRAF6 which was further enhanced by LPS, though biologically relevant (>2 fold regulation) only for TRAF6. In contrast to the GalN+LPS model, treatment with TAK-242 in the BDL+LPS model reverted the down-regulatory effect of MyD88 and TRAF6.

Therefore, chronic liver injury is characterized by hepatic TLR4 receptor over-expression but compensatory down-regulation of TLR4 signaling regulator genes and thus limited hepatic cytokine activation in response to LPS injection. In contrast, if ALF is induced by GalN+LPS in animals with normal liver without preconditioned TLR4 pathway, the cytokine expression is dramatically increased.

Discussion

The results of this study provide the scientific evidence to translate novel insights into the pathogenesis of multiorgan dysfunction in ACLF and ALF for the treatment of these conditions, both associated with high-risk of death. TLR4 is a key pattern recognition receptor (PRR) for endotoxins and cell death molecules mediating pro-inflammatory signals in parenchymal and non-parenchymal cells that have been shown to be elevated in ACLF and ALF providing rationale for targeting this receptor (28, 29). The data presented here show in cell models, 2 animal models of ACLF and one model of ALF, that the administration of TAK-242, a specific inhibitor of the TLR4 receptor reduces the severity of inflammation, hepatocyte cell death and improves organ function, arguing strongly to test this molecule in clinical trials.

In previous studies of rodent models of cirrhosis, an exquisite sensitivity to LPS has been demonstrated (6). Administration of LPS to cirrhotic animals resulted in the rapid development of severe liver and multiorgan failure whilst naïve animals tolerated equivalent doses extremely well (6). In the present study, we confirmed that the expression of the endotoxin receptor, TLR4, was significantly increased in hepatocytes in biopsies from humans with cirrhosis and rodent models, which may be the mechanism underlying the significant endotoxin sensitivity. In patients with acute decompensation of cirrhosis, numerically higher plasma levels of both circulating cleaved nucleosomes and LPS were detected, but did not reach statistical significance, unlike in previous studies(4). However, plasma samples, when exposed to HEK-TLR4 reporter cell line, induced strong TLR4 transactivation suggesting a significant increase of bioactive TLR4 ligands in the plasma of these patients. The pathophysiological relevance of this observation is exhibited in the *in vitro* studies demonstrating both LPS-induced cell death of

immortalized hepatocytes and enhanced cytokine release in immortalized monocytes. These deleterious effects of LPS were effectively abrogated by TAK-242, a selective inhibitor of TLR4, providing evidence for the importance of this receptor in mediating the LPS induced effects. Taken together, these data support the hypothesis that it is not the quantity of the TLR4 ligands in the circulation that results in ACLF but the expression of TLR4 in the epithelial cells of various organs.

TLR4 is an attractive therapeutic target for patients with liver diseases as it is involved in several inflammatory and fibrotic processes and regulates the degree of liver fibrosis induced by various conditions such as non-alcoholic steatohepatitis but also in animal models of chronic liver injury (BDL or CCl₄ models) (30-32). TLR4 is also expressed on numerous subsets of immune cells, such as Kupffer and dendritic cells, which are also present in the liver (33) and they are able to release strong pro-inflammatory signals upon activation (34), an important mechanism for tissue injury in ACLF (25). A first attempt to translate TAK-242 into humans with sepsis reached Phase 3 levels and confirmed the safety of the drug but failed to reach the primary end-point of survival. In patients with sepsis due to gram-negative infection, severe sepsis and respiratory failure, a trend to improved survival was observed (35). The pathophysiological backdrop of ACLF, makes TLR4 an attractive therapeutic target that led to exploration of its role in the animal models.

TAK-242, when used in the BDL model of ACLF significantly abrogated liver, brain and kidney injury and dysfunction, in both the prophylactic and therapeutic modes of administration. The clinical observations were striking as the coma-free mortality of the animals could be reduced by

100% and about 50% in the groups treated with TAK-242 administered prophylactically and when TAK-242 was administered in the treatment mode. These observations were confirmed in the carbon-tetrachloride mouse model. These data extend previous studies, which showed reduced liver injury with TAK-242 in BDL animals, those with ischemia-reperfusion injury and our previous observation that TLR4 knock out mice were protected from developing multi-organ injury secondary to acetaminophen-induced ALF compared to wild type mice (18). We therefore assessed the efficacy of TAK-242 to also prevent multi-organ dysfunction in another rat model where ALF was induced by GalN+LPS treatment and showed that TAK-242 significantly abrogated the liver injury. Taken together the data provide evidence of efficacy of TAK-242 both for ACLF and also ALF.

Although it is clear that LPS can cause hepatocyte cell death and also activate inflammatory cells as shown in this study, it is important to note that there was a clear distinction between the acute and chronic model with respect to TLR signaling pathway modulation and cell death mode. In both ALF and ACLF, extensive areas of liver cell death were shown using TUNEL staining. However, it is intriguing to notice that *in vivo* TLR4 signaling leads to different types of hepatocyte cell death depending on the presence or absence of chronic liver injury. In the ACLF model, plasma RIPK3 levels were increased and caspase 3/7 activity decreased, which defines a necroptotic cell death whereas in GalN/LPS treated rats, apoptosis with high caspase 3/7 and low RIPK3 levels was the predominant mode of cell death. The fact that TLR4 inhibition clearly reversed the LPS response (36) incontrovertibly, suggests that the TLR4 pathways is an important mediator of both cell death modes in these models. In the ACLF model, hepatic TNF α , MyD88, TRAF6 and caspase 8 activity were downregulated suggesting a TNF α independent

mechanism alleviating necroptosis. Binding of TNF α leads to formation of RIPK1-RIPK3 complex (necrosome) leading to necroptosis in case caspase 8 is absent. Mixed lineage kinase domain-like pseudokinase (MLKL) mediates pore formation and Ca²⁺ influx leading to cell swelling and subsequent cell death (37-41). However, ligand binding to TLR4 can lead to an RIPK1-independent (TNF α independent) activation of RIPK3 via the RIM domain of the regulator protein TRIM. This possibly explains why although TNF α is downregulated in the BDL model, superimposed LPS leads to high grade hepatic necroptosis. In animal studies, RIPK1 inhibition by necrostatin was shown to be effective to prevent liver tissue necroptosis (42), which is the predominant cell death mode in the ACLF model but not ALF model. Its important to mention that in contrast to the liver, circulating TNF α levels along with other pro-inflammatory cytokines were significantly increased after LPS injection. This may be due to cytokine release by immune cells such as monocytes and tissue resident macrophages, which are sensitized in patients with cirrhosis alleviating cytokine release and burst reactions (43, 44). Importantly both effects could be effectively reversed by TLR4 inhibition with TAK-242.

The main limitation of this study is that the precipitating event used to cause ACLF and ALF in the animal models was LPS, hence the models do not cover the entire spectrum of ACLF and ALF pathophysiology. Nevertheless, the BDL model is clinically relevant as it mimics secondary biliary cholangitis and bacterial infection is the most important precipitating event of ACLF. As we have already shown potential role of TLR4 antagonism in acetaminophen-induced ALF previously, we chose to use GalN/LPS model as it allowed us to explore the different mechanisms of injury and beneficial effect of TAK-242 operating in the ACLF and ALF.

In conclusion, the data presented in this study provide strong evidence of the efficacy of TAK-242 to treat ACLF and possibly also ALF in clinically relevant rodent models that can be translated for the benefit of patients. As TAK-242 has proven safety record, it could be repurposed for clinical trials in patients with ACLF and ALF.

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Acknowledgments: We would like to thank Harry Horsley (Center of Urological Biology, University College London) for his support to perform the confocal microscopy.

Figures and figure legends

Figure 1: Toll-like receptor 4 in human samples and in vitro studies.

Sensitization to TLR4 ligands and high levels of pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) are potential factors explaining the high susceptibility in cirrhosis to events leading to ACLF. A) Liver TLR4 expression was evaluated by DAB immunohistochemistry in human liver biopsies (healthy controls n=2, alcoholic liver disease n=12, chronic hepatitis B n=7). LPS (PAMP) and nucleosomes (DAMP) were measured in plasma of human cohorts (healthy controls n=10; compensated cirrhosis n=17; acute decompensated cirrhosis n=151). B) The circulating LPS and cleaved nucleosome concentration increased numerically in patients with acute decompensation but did not reach statistical significance. The ability of circulating TLR4 ligands to transactivate TLR4 signaling was assessed by HEKBlue-hTLR4 reporter cell. Cell response was significantly higher after incubation with plasma from patients with acute decompensated cirrhosis compared to healthy controls (p<0.001). C) In liver tissue from patients with cirrhosis, both in alcoholic liver disease and chronic hepatitis B, TLR4 expression was increased, reaching statistical significance in Hepatitis B compared to healthy controls (p=0.001) and alcoholic liver disease (p<0.001). D) The effect of TLR4 inhibition with TAK-242 was tested in two different cell lines, immortalized human monocytes (THP1) (unstimulated and PMA stimulated) and immortalized human hepatocytes (HHL5). In THP1 cells LPS response was assessed by IL6 mRNA expression analysis 5 hours after LPS incubation and in HHL5 by TUNEL staining and caspase 3/7 enzyme activity. E) Both types of THP-1 cells (monocytes and PMA stimulated macrophages) showed an increase in IL6 mRNA expression after LPS incubation. This effect was prevented by TAK-242. Upon LPS stimulation, an increasing number of HHL5 cells were apoptotic (TUNEL staining) with associated high levels of caspase 3/7 enzyme activity

stimulation, both prevented by TAK-242. Analyses were performed with SPSS 22, one-sided ANOVA with Tukey post-hoc analysis for multiple comparison correction.

Figure 2: TLR4 inhibition by TAK-242 to treat ACLF

As TLR4 driven sensitization to endotoxins is a potential mediator of organ injury in ACLF, we tested whether TLR4 inhibition by TAK-242 is effective in preventing multi-organ failure. Two animal models mimicking ACLF were evaluated. A) Rats were bile duct ligated (BDL) for 4 weeks (n=7-16 per group, see also Material and Methods) and D) mice were gavaged with carbon tetrachloride (CCl₄) for 6 weeks (n=4 per group), followed by i.p. lipopolysaccharide (LPS) injection as second hit. In the BDL model TAK-242 was injected prophylactically (30 min before LPS) and therapeutically 45 min after LPS. Mice received TAK-242 twice, 45 min and 22 hours after LPS injection. B) In rats with BDL, mortality dropped to 13% after injection of LPS but improved markedly to 100% after TAK-242 injected prophylactically and 53% with TAK-242 in therapeutic administration. The mean arterial pressure improved numerically after TAK-242 injection. C) H&E staining showed an advanced chronic liver injury with regenerative areas after BDL in rats. There was a marked liver injury after LPS injection with vast areas of cell death and high ALT levels. Both were significantly reduced by treatment with TAK-242. Acute kidney injury (TUNEL staining, Creatinine levels) was abrogated by either prophylactic or therapeutic TAK-242 treatment. E) After 6 weeks of CCl₄ gavage mice developed bridging liver fibrosis (Sirius red staining). After LPS injection the liver showed inflammatory cell infiltrates (H&E staining), cell death (TUNEL staining) and increased ALT levels. The liver injury was diminished after 24 hours treatment with TAK-242. F) LPS driven renal tubular cell death was inhibited by TAK-242. Analyses were performed with SPSS 22, one-sided ANOVA with Tukey post-hoc analysis for multiple comparison correction.

Figure 3: TLR4 inhibition by TAK-242 to treat ALF.

A) Study outline for testing the efficacy of TAK-242 in Galactosamine+LPS (GalN+LPS) induced acute liver failure (Vehicle n=10, GalN n=5, GalN + LPS n=10, GalN+LPS+TAK-242 n=7). B) The liver injury was marked after GalN+LPS. ALT levels and TUNEL positive areas in liver tissue increased significantly ($p<0.001$, $p<0.001$) and albumin levels decreased ($p=0.034$). TAK-242 ameliorated cell death ($p<0.001$) proving the positive treatment effect on liver injury in this model. Although GalN+LPS was accompanied by apoptotic cell death in kidney tissue ($p=0.027$), plasma creatinine levels were unchanged. TAK-242 improved kidney cell death, however statistically not significant. C) GalN+LPS led to a systemic inflammatory response with increasing IL1 β concentrations ($p<0.001$). IL1 β cytokine levels responded significantly to TAK-242 ($p<0.001$). Other plasma cytokines such as IL6, TNF α and IL10 showed the same trend without reaching significance. Analyses were performed with SPSS 22, one-sided ANOVA with Tukey post-hoc analysis for multiple comparison correction.

Figure 4 TLR4 pathway modulations:

TLR4 is expressed on hepatocytes and potentially mediates directly LPS related cellular injury. Therefore, we assessed the effect of liver injury and TAK-242 treatment on the TLR4 signaling pathway. A) Immunohistochemistry showed hepatic TLR4 overexpression in response to chronic liver injury in the BDL and CCl₄ model. Treatment with TAK-242 in the BDL model did not alter TLR4 overexpression whereas prolonged treatment (24h) in the CCl₄ model

induced TLR4 down-regulation in the liver. B) mRNA was isolated from liver tissue and the expression of 84 TLR related genes was measured by qPCT (mRNA profiler). Five samples per group were pooled to be tested on one profiler plate. The heatmap consists of a color-coded depiction of mRNA genes regulations (red – upregulation, blue – down-regulation). It is apparently, that both, the GalN model and BDL model were associated with different TLR pathway activation patterns. C) Individual qPCR were performed in individual samples for the most prominent TLR4-related genes: MyD88, TRAF6, TNF α and IL6. Relative gene expression was analyzed by using the ddCt method and delineated in a log2 scale. A 2 fold up-regulation or 0.5 down-regulation was considered as biologically significant. The regulatory gene of the TLR4 signaling pathway, TRAF6, showed a lower expression in response to liver injury in both models. BDL related down-regulation of TRAF6 was associated with low expression of TNF α and IL6 in response to LPS, whereas GalN led to a marked increase of TNF α and IL6 mRNA levels. Analyses were performed with SPSS 22, one-sided ANOVA with Tukey post-hoc analysis for multiple comparison correction.

A

B

C

D

F

E

A

B

Coma free survival rate in rats after BDL

Mean arterial pressure in rats after BDL

C

D

E

F

A **B** **C**

A

B

C