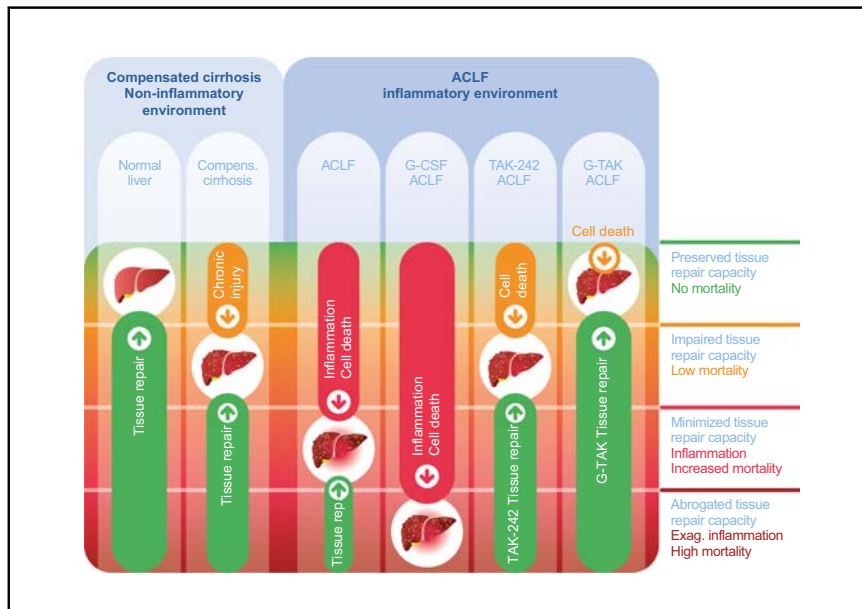


# Combination of G-CSF and a TLR4 inhibitor reduce inflammation and promote regeneration in a mouse model of ACLF

## Graphical abstract



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## Lay summary

Acute-on-chronic liver failure is associated with severe liver inflammation and poor short-term survival. Therefore, effective treatments are urgently needed. Herein, we have shown, using mouse models, that the combination of granulocyte-colony stimulating factor (which can promote liver regeneration) and TAK-242 (which inhibits a receptor that plays a key role in inflammation) could be effective for the treatment of acute-on-chronic liver failure.

## Highlights

- We hypothesised that a TLR4 inhibitor, TAK-242, might mitigate the negative effects of G-CSF in ACLF.
- G-CSF alone increases mortality and promotes inflammation in rodent models of ACLF.
- The combination of TAK-242 and G-CSF inhibits inflammation, promotes hepatic regeneration and prevents mortality in models of ACLF.



# Combination of G-CSF and a TLR4 inhibitor reduce inflammation and promote regeneration in a mouse model of ACLF

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**Background & Aims:** Acute-on-chronic liver failure (ACLF) is characterised by high short-term mortality, systemic inflammation, and failure of hepatic regeneration. Its treatment is a major unmet medical need. This study was conducted to explore whether combining TAK-242, a Toll-like receptor-4 (TLR4) antagonist, with granulocyte-colony stimulating factor (G-CSF), could reduce inflammation whilst enhancing liver regeneration.

**Methods:** Two mouse models of ACLF were investigated. Chronic liver injury was induced by carbon tetrachloride; lipopolysaccharide (LPS) or galactosamine (GalN) were then administered as extrahepatic or hepatic insults, respectively. G-CSF and/or TAK-242 were administered daily. Treatment durations were 24 hours and 5 days in the LPS model and 48 hours in the GalN model.

**Results:** In a mouse model of LPS-induced ACLF, treatment with G-CSF was associated with significant mortality (66% after 48 hours vs. 0% without G-CSF). Addition of TAK-242 to G-CSF abrogated mortality (0%) and significantly reduced liver cell death, macrophage infiltration and inflammation. In the GalN model, both G-CSF and TAK-242, when used individually, reduced liver injury but their combination was significantly more effective. G-CSF treatment, with or without TAK-242, was associated with activation of the pro-regenerative and anti-apoptotic STAT3 pathway. LPS-driven ACLF was characterised by p21 overexpression, which is indicative of hepatic senescence and inhibition of hepatocyte regeneration. While TAK-242 treatment mitigated the effect on senescence, G-CSF, when co-administered with TAK-242, resulted in a significant increase in markers of hepatocyte regeneration.

**Conclusion:** The combination of TAK-242 and G-CSF inhibits inflammation, promotes hepatic regeneration and prevents

mortality in models of ACLF; thus, this combination could be a potential treatment option for ACLF.

**Lay summary:** Acute-on-chronic liver failure is associated with severe liver inflammation and poor short-term survival. Therefore, effective treatments are urgently needed. Herein, we have shown, using mouse models, that the combination of granulocyte-colony stimulating factor (which can promote liver regeneration) and TAK-242 (which inhibits a receptor that plays a key role in inflammation) could be effective for the treatment of acute-on-chronic liver failure.

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## Introduction

Acute-on-chronic liver failure (ACLF) is characterised by multi-organ failure in hospitalised patients with acute decompensation of cirrhosis.<sup>1</sup> The 90-day mortality rates vary from 30-100% depending on the age, number of organ failures and the severity of systemic inflammation. Systemic and hepatic inflammation,<sup>2</sup> non-apoptotic cell death<sup>3-5</sup> and a lack of hepatic regeneration<sup>6</sup> are major factors determining the development, course and prognosis of ACLF. Damage-associated molecular patterns (DAMPs) of both nuclear [core histones, high mobility group box-1 (HMGB1)] and cytoskeletal (cytokeratin 18, K18) origin are released by injured tissues where cell death is prevalent.<sup>5,7,8</sup> Furthermore, circulating pathogen-associated molecular patterns (PAMPs), of which lipopolysaccharide (LPS) is a key player<sup>9</sup> are also increased as a result of infections and/or bacterial translocation from the gut into the systemic circulation. Binding of DAMPs and PAMPs to their cognate pattern recognition receptors, predominantly Toll-like receptor 4 (TLR4), triggers NF- $\kappa$ B-dependent transcription and secretion of pro-inflammatory cytokines, such as IL1 $\beta$ , IL6 and TNF $\alpha$  leading to a cascade of inflammation and cell death.<sup>8,10</sup> Targeted inhibition of TLR4 with a small molecule inhibitor, TAK-242, has been shown to reduce the short-term mortality of animals with ACLF<sup>4</sup> but its administration is associated with a reduction in hepatocyte regeneration.<sup>11</sup>

Keywords: liver failure; stem cell therapy; systemic inflammation; regeneration; senescence.

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Lack of hepatic regeneration is a feature of ACLF and, indeed, hepatocyte proliferation is a positive predictor of survival.<sup>12,13</sup> Hepatocellular senescence has been hypothesised to inhibit hepatic regeneration in cirrhosis<sup>14</sup> and hepatic inflammation is thought to be the main cause of inhibited cell cycle progression.<sup>13,15</sup> To modulate hepatic regeneration in ACLF, granulocyte-colony stimulating factor (G-CSF), which mobilises bone marrow-derived stem and immune cells has been studied extensively in patients with ACLF. In several small, underpowered studies in patients with decompensated cirrhosis, alcoholic hepatitis and ACLF, G-CSF was shown to improve survival and was associated with mobilisation of CD34<sup>+</sup> stem cells.<sup>16–19</sup> However, these results could not be reproduced in a large, placebo-controlled multicentre study in Germany.<sup>20</sup> Additionally, about twice the number of G-CSF-treated patients developed ACLF during follow-up compared with the placebo group. Moreover, 7 serious adverse reactions were observed in the G-CSF-treated group; in 5 this was related to aggravated organ failure.<sup>20</sup> Previous single-centre trials, using various dosing regimens ranging from 5 to 26 days with doses of 5 µg/kg once daily to 300 µg twice daily, reported similar beneficial results independent of the treatment scheme. The fact that the 2 multicentre trials covered a similar spectrum with short high-dose treatment (15 µg/kg once daily<sup>21</sup>) and rather low-dose long-term therapy (5 µg/kg<sup>20</sup>) speaks against the argument that the lack of efficacy was dose or timing related.

This study was designed to test the hypothesis that the lack of beneficial effect of G-CSF was possibly due to worsening of systemic inflammation through simultaneous release of bone marrow-derived inflammatory cells. We further hypothesised that this increased inflammation was through a TLR4-dependent mechanism, as previously shown in a rat model of partial hepatectomy.<sup>23</sup> The aims of this study were to determine whether the addition of TAK-242, a TLR4 antagonist, would prevent G-CSF-related aggravation of liver injury and enable hepatic regeneration. We also aimed to better define the underlying mechanisms of the interplay between G-CSF and TAK-242.

## Material and methods

### Study design

This study explored the hypothesis in 2 mouse models of ACLF. All animals were included in the analysis unless specified in the figure legends. Animals were randomly assigned to experimental interventions, but no blinding was applied. All experiments were performed and reported according to the ARRIVE guidelines.<sup>24</sup>

### Animal models

All animal experiments were performed 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. After intervention, animals were monitored closely and prematurely sacrificed if pre-comatose and/or if the disease condition rapidly worsened. Animals in each group were terminated by exsanguination under general anaesthesia with isoflurane (2% isoflurane in oxygen, Piramal Healthcare, USA).

#### Carbon tetrachloride-LPS model

Male C57BL/6 mice (body weight: 30 g±4 g; age:8-10 weeks) were gavaged with carbon tetrachloride (CCl<sub>4</sub> 0.5 mg/ml dissolved in olive oil – dose 0.5 ml/kg bw) twice weekly for a total of 6 weeks to induce liver fibrosis, as described previously.<sup>25</sup> To mimic ACLF, LPS (*Klebsiella pneumoniae*, Sigma, UK), dissolved in

saline to a final concentration of 6.25 µg/µl was injected i.p to a final dose of 4 mg/kg. Therapeutic interventions with G-CSF (250 µg/kg, s.c.) and/or TAK-242 (10 mg/kg, i.p.) were started 1 hour after LPS injection and repeated either 22 hours after LPS injection in the 24-hour model or daily in the 5-day model, respectively. Animals were sacrificed 24 hours or 5 days after LPS injections and 2 hours after the last therapeutic intervention. Time points were chosen based on our previous studies where we observed that there was significant liver injury 24 hours after LPS injection in CCl<sub>4</sub> pre-treated animals.<sup>4</sup> Liver regeneration and hepatocyte proliferation reaches its maximum earliest after 72 hours, with re-established liver mass after 7 days,<sup>26</sup> so that the second time point was chosen at 5 days after LPS injection.

The study groups were as follows: Vehicle (olive oil, saline) (n = 10); CCl<sub>4</sub> (n = 10); CCl<sub>4</sub>+G-CSF (5 days n = 10); CCl<sub>4</sub>+LPS (24 hours n = 10; 5 day n = 6); CCl<sub>4</sub>+LPS+G-CSF (24 hours n = 10; 5 days n = 6); CCl<sub>4</sub>+LPS+TAK-242 (24 hours n = 4; 5 days n = 6); CCl<sub>4</sub>+LPS+TAK-242+G-CSF (24 hours n = 4; 5 days n = 10).

Vehicle injections were performed with olive oil for CCl<sub>4</sub>, saline for LPS, saline for G-CSF and citric acid/NMP/Captisol for TAK-242.

#### CCl<sub>4</sub>-Galactosamine model

Male C57BL/6 mice (n = 8-10 per group) (body weight: 30 g±5 g; age: 8-10 weeks) were gavaged with CCl<sub>4</sub> (0.5 mg/ml dissolved in olive oil – dose 0.5 ml/kg) twice weekly for a total of 6 weeks to induce liver fibrosis. Galactosamine (GalN, Sigma, UK), dissolved in saline, was injected i.p. (1,000 mg/kg) to induce liver injury. Therapeutic interventions with G-CSF (250 µg/kg, s.c.) and/or TAK-242 (10 mg/kg, i.p.) were started 1 hour after GalN injection and repeated after 22 hours and 46 hours post GalN injection. For RIPK1 inhibition, RIPA56 (3 mg/kg, i.p.) was given 1 hour after GalN and continued twice daily until animals were terminated.

The study groups were as follows: Vehicle (n = 10); CCl<sub>4</sub> (n = 10); CCl<sub>4</sub>+GalN (n = 8); CCl<sub>4</sub>+GalN+RIPA56 (n = 8); CCl<sub>4</sub>+GalN+G-CSF (n = 8); CCl<sub>4</sub>+GalN+TAK-242 (n = 8); CCl<sub>4</sub>+GalN+TAK-242+G-CSF (n = 8).

Vehicle injections were performed with olive oil for CCl<sub>4</sub>, saline for GalN, saline for G-CSF and citric acid/NMP/Captisol for TAK-242.

### Sampling and storage

Blood samples were taken from the right heart. Lithium heparin plasma was centrifuged 2,500 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, for histological assessments, a sample of the organs was formalin-fixed (10% neutral buffered saline, Leica Biosystems, UK) for 24 hours before paraffin-embedding.

### Statistical analysis

The sample size was calculated based on the results provided by Theocharis *et al.*<sup>27</sup> showing an alanine aminotransferase (ALT) reduction from 3,674 U/L ± 450 to 2,450 U/L ± 225 after treatment with recombinant G-CSF in mice with thioacetamine-induced acute liver failure. We hypothesised conservatively an effect size of 15% in our model with alpha error of 0.05, providing a power of 80% for samples of 7 animals per group and 90% for samples of 10 animals per group. The sample size was confirmed by using data from an ACLF rat model of bile duct ligation and LPS injection where LPS injection increased ALT levels to 129.4 ±

33 U/L whilst TAK-242 pre-treatment reduced it to  $66.2 \pm 9.4$  U/L.<sup>4</sup> By using an alpha error of 0.05, we calculated that 8 animals per group were needed for a power of 80% and 12 animals per group for a power of 90%. Finally, we aimed at having 10 animals per group unless stated otherwise.

All statistical analyses were performed using SPSS 24 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-squared test. If more than 2 groups had to be analysed, one-sided ANOVA with *post hoc* Tukey analyses for multiple comparison were used. A *p* value  $\leq 0.05$  was considered statistically significant. Graphs were prepared in Prism (GraphPad, USA) and figures compiled in Adobe Photoshop (Adobe Systems, USA).

For further details regarding the materials and methods used, please refer to the [CTAT table](#) and [supplementary information](#).

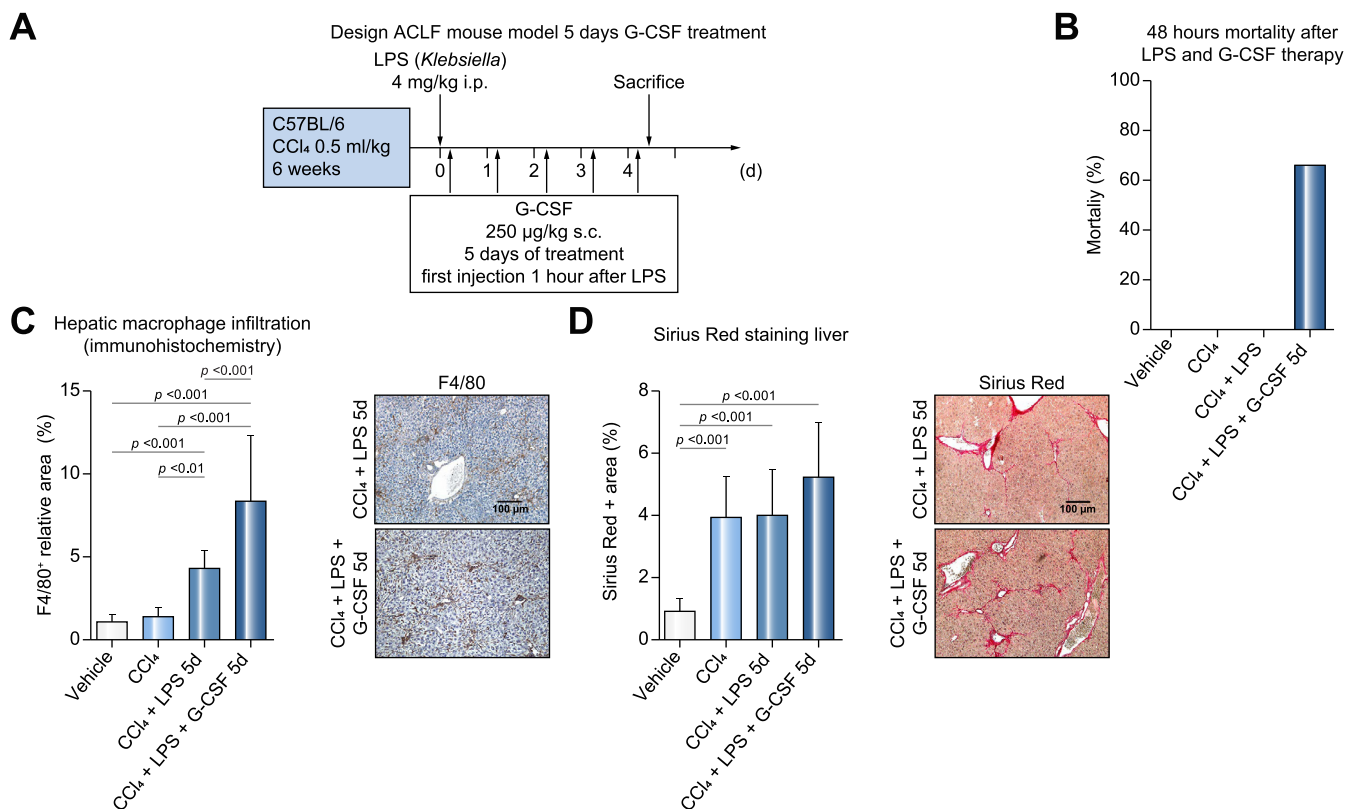
## Results

### G-CSF increases the mortality of rodents with ACLF

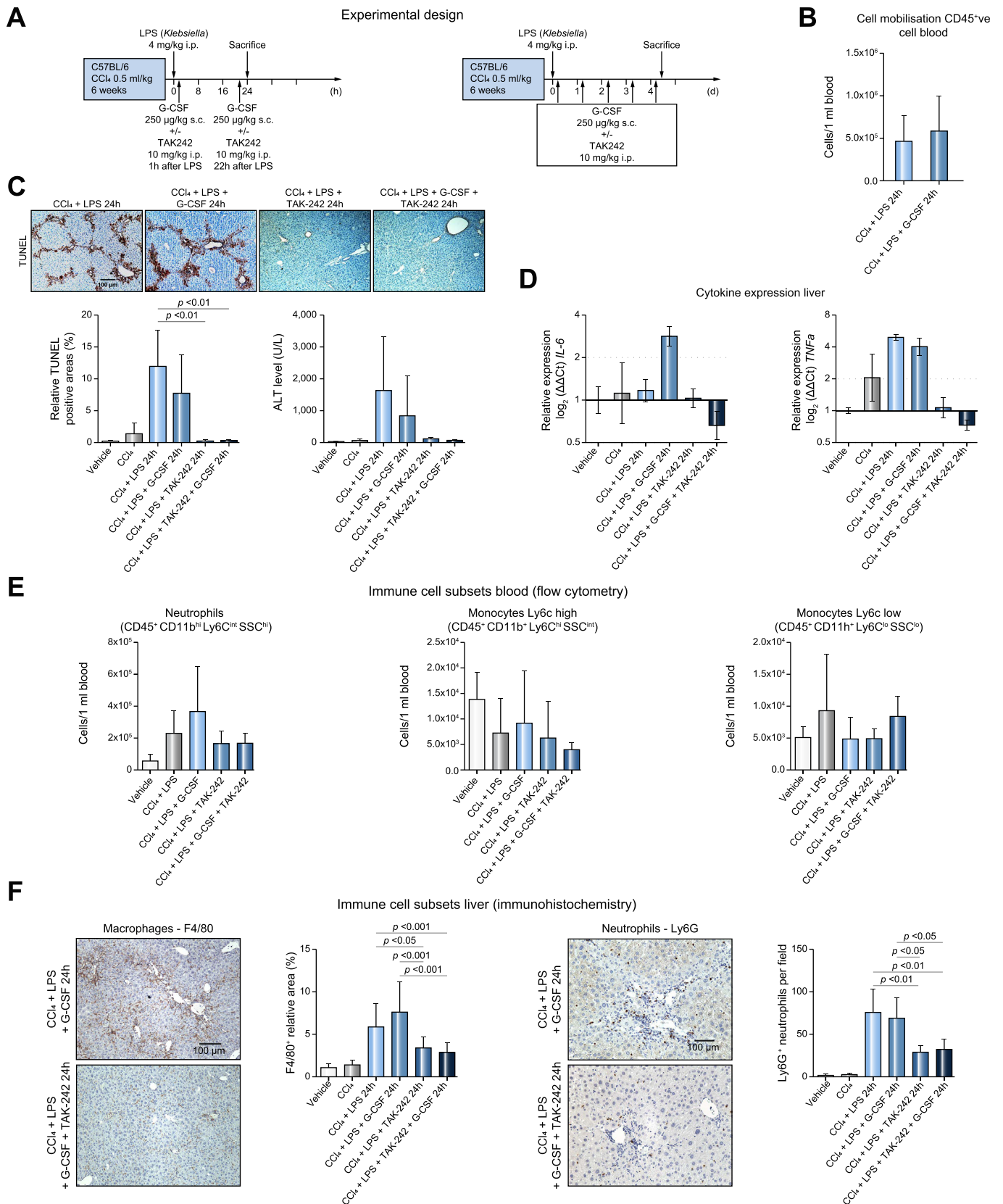
To understand the pathophysiological basis of the lack of benefit and possible deleterious effect of G-CSF treatment that was observed in the German clinical trial (GRAFT study),<sup>20</sup> we performed preclinical studies in murine models of ACLF. In the first model, mice were gavaged for 6 weeks with CCl<sub>4</sub> and then injected with LPS to induce liver injury, as described

previously<sup>4,25</sup> (Fig. 1A). Six-week CCl<sub>4</sub> administration induced bridging fibrosis (Fig. 1D). This model mimics the typical features consisting of multi-organ injury in response to administration of LPS and pre-existing chronic liver injury.<sup>4,25</sup> Twenty-four hours after LPS injection these animals developed significant liver and kidney injury with high ALT and creatinine levels and extended areas of hepatocyte cell death (TUNEL staining) (Fig. 2, Fig. S1). Hepatic expression of CCL5, ICAM-1 and NGAL, as markers of inflammation and organ injury, also increased after LPS injection compared to vehicle (*p* < 0.001) (Fig. S2).<sup>4</sup> Liver injury, measured by ALT levels and liver cell death (TUNEL), was decreased 5 days after LPS injection (Fig. S1).

One-hour after LPS injection, mice were treated with G-CSF subcutaneously (250 µg/kg) once daily for 5 consecutive days. Twenty-four hours after the first G-CSF injection the number of mobilised CD45<sup>+</sup> cells in the blood increased from a median of 321,900 cells/ml blood (CCl<sub>4</sub>+LPS) to 510,504 cells/ml (CCl<sub>4</sub>+LPS+G-CSF) (Fig. 2B). G-CSF therapy led to a mortality rate of 66% after 48 hours (Fig. 1B) and a high degree of liver fibrosis (Sirius Red) (*p* < 0.001, compared to vehicle) (Fig. 1D), whereas all other animals without G-CSF treatment survived the 5-day follow-up. G-CSF administration was associated with increased infiltration of the liver with macrophages (F4/80<sup>+</sup>-DAB-positive areas [CCl<sub>4</sub>+LPS] 4.3 % ± 1.1 vs. 8.4 % ± 3.9 [CCl<sub>4</sub>+LPS+G-CSF]; *p* < 0.001) (Fig. 1C) after 5 days of G-CSF treatment. Therefore, in



**Fig. 1. Effect of G-CSF in an ACLF mouse model.** (A) Animals were gavaged for 6-weeks with 0.5 ml/ml CCl<sub>4</sub> to induce chronic liver injury. Thereafter, LPS (*Klebsiella pneumoniae*) was injected to induce multi-organ injury. G-CSF (250 µg/kg, s.c.) was injected 1 hour after LPS and continued for 5 days once daily (Vehicle *n* = 10, CCl<sub>4</sub> *n* = 10, CCl<sub>4</sub> + LPS *n* = 6, CCl<sub>4</sub> + LPS + G-CSF *n* = 6). (B) In total, 66% of all animals died in the group treated with G-CSF alone. Survival in the other groups was 100%. (C) 5 days of G-CSF treatment increased hepatic macrophage infiltration (F4/80<sup>+</sup>) compared to animals with CCl<sub>4</sub>+LPS only (*p* < 0.001). (D) This ACLF model was associated with significant collagen accumulation (Sirius Red staining, *p* < 0.001 compared to vehicle) after 5-days CCl<sub>4</sub>. Group comparison was performed by one-way ANOVA and *post hoc* Tukey's multiple comparison. *p* > 0.05 was considered statistically significant. ACLF, acute-on-chronic liver failure; CCl<sub>4</sub>, carbon tetrachloride; G-CSF, granulocyte-colony stimulating factor; LPS, lipopolysaccharide. (This figure appears in color on the web.)



**Fig. 2. TAK-242 prevents the deleterious effect of G-CSF in the CCl<sub>4</sub>-LPS model.** (A) C57BL/6 mice were gavaged for 6-weeks with CCl<sub>4</sub> 0.5 mg/ml to induce chronic liver injury. Thereafter, LPS (*Klebsiella pneumoniae*) was injected to induce multi-organ injury. G-CSF (250 µg/kg, s.c.) was injected 1 hour after LPS and continued for 24 hours or 5 days once daily. TAK-242 was administered concurrently (10 mg/kg, i.p.) and continued once daily for 24 hours or 5 days. (B) G-CSF

this model, G-CSF aggravated ACLF-related mortality, which was associated with worsening of hepatic macrophage infiltration.

#### TLR4 inhibition by TAK-242 prevents G-CSF-related mortality and inflammation

As our previous studies have shown increased hepatocyte TLR4 expression being involved in organ sensitisation to LPS in cirrhosis and a reduction in the severity of inflammation with inhibition of TLR4 in models of ACLF,<sup>3</sup> we hypothesised that the deleterious effect of G-CSF in ACLF may be modulated favourably by TLR4 inhibition.<sup>28</sup> We therefore used TAK-242, a small molecule TLR4-inhibitor, with or without G-CSF in the CCl<sub>4</sub> – LPS model over 24 hours and 5 days (Fig. 2A).

Administration of TAK-242 together with G-CSF reduced the mortality rate from 66%, in G-CSF-treated animals, to 0% (Fig. S10). Liver cell death was measured by TUNEL staining 24 hours after LPS injection. The median relative TUNEL-positive area in liver tissue increased from 0.7% (range 0.2–3.3) to 10.1% (range 0.3–22.1), which was not significantly different to the 7.8% (range 1.6–22.1) observed when G-CSF was injected after LPS. Treatment with TAK-242, alone or in combination with G-CSF, significantly reduced the TUNEL-positive area to 0.19% (range 0.06–0.55,  $p < 0.01$ ) and 0.27% (range 0.16–0.5,  $p < 0.01$ ), respectively (Fig. 2C), and reduced ALT levels (Fig. 2C). The STAT3 pathway reduces apoptosis through release of B-cell lymphoma 2 protein (BCL2), which antagonises BCL2-associated X protein (BAX). The protective effect of TAK-242/G-CSF was associated with activation of the STAT3 pathway (increased pSTAT3) (Fig. S3), possibly induced by IL-22 secretion (Fig. S2E), with increased expression of anti-apoptotic BCL2 and higher BCL2/BAX ratio (Fig. S3). Likewise, injury markers (such as Lipocalin/NGAL and IL-13) were increased with G-CSF but reduced if TAK-242 was added, as shown by the cytokine/chemokine protein arrays (Fig. S2B/C).

Inflammatory response was assessed by measuring liver cytokine expression at both the mRNA and protein level. LPS injection led to >4-fold increase in hepatic *TNF $\alpha$*  mRNA expression, whereas treatment with G-CSF induced an increase in *IL-6* mRNA expression level (>2 fold), which was markedly reduced by TAK-242, with or without G-CSF (Fig. 2D). G-CSF increased the liver protein expression of inflammatory markers such as CXCL9, LIF and Petraxin3/TSG-14 as shown by the protein array data from pooled liver lysates. These markers were reduced after TAK-242 injection (Fig. S2). TAK-242 treatment resulted in reduced hepatic infiltration of F4/80<sup>+</sup> macrophages (DAB-positive area [%] – 7.6%±3.6 [CCl<sub>4</sub>+LPS+G-CSF] vs. 2.9%±1.2 [CCl<sub>4</sub>+LPS+G-CSF+TAK-242],  $p < 0.001$ ) and Ly6G<sup>+</sup> neutrophils (Ly6G positive cells per bright field – 68.9±1.2 [CCl<sub>4</sub>+LPS+G-CSF] vs. 32.3±12.7 [CCl<sub>4</sub>+LPS+G-CSF+TAK-242],  $p < 0.05$ ) (Fig. 2F). High numbers of circulating neutrophils and Ly6c-high monocytes occurred in the blood after G-CSF therapy. Their numbers decreased with TAK-

242 therapy and the combination of TAK-242 and G-CSF led to an increase of Ly6c-low monocytes (Fig. 2E). LPS injection was also associated with stellate cell activation ( $\alpha$ SMA expression), which was significantly abrogated by TAK-242 (Fig. S4).

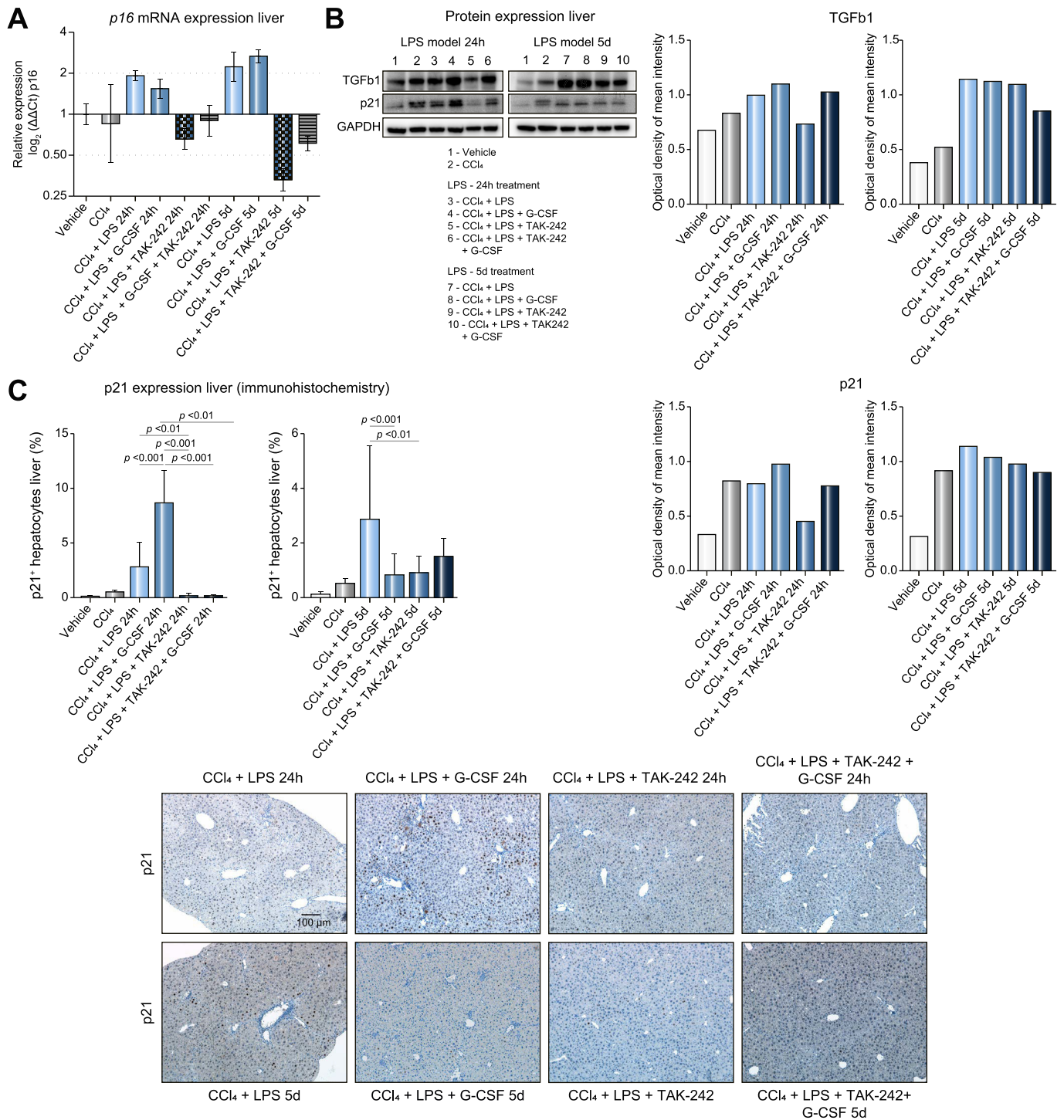
Monocytes and macrophages are major sources of cytokine release in liver disease.<sup>29</sup> We therefore tested to what extent LPS and G-CSF modulate the cytokine response in PMA (phorbol 12-myristate-13-acetate)-activated (*macrophage-like*) and naïve THP1 cells (*monocyte-like*) *in vitro*. The experiments showed that G-CSF incubation of PMA-activated THP1 cells and naïve THP1 cells prior to LPS stimulation resulted in an aggravated cytokine response, especially upregulation of *IL6* mRNA expression (THP1 macrophages+LPS [10 ng/ml]: 6.2-fold upregulation vs. LPS [10 ng/ml]+G-CSF [100 ng/ml]: 6.7-fold upregulation; THP1 monocytes+LPS [10 ng/ml]: 60.2-fold upregulation vs. LPS [10 ng/ml]+G-CSF [100 ng/ml]: 71-fold upregulation [Fig. S5A]). Results were confirmed in primary peripheral blood mononuclear cells isolated from healthy donors and incubated with pooled plasma from 9 patients with ACLF (Table S3). These cells exhibited a strong upregulation of IL-1b after incubation with G-CSF prior to LPS, compared to cells incubated with LPS alone. TAK-242 prevented excess cytokine production in peripheral blood mononuclear cells with or without G-CSF (Fig. S5B). These *in vitro* findings are in keeping with hepatic IL6 levels, which increased 2.8-fold in G-CSF-treated animals (Fig. 2D). These changes, either with LPS alone or in combination with G-CSF, were completely prevented in the presence of TAK-242, clearly showing that TAK-242 prevents the overwhelming inflammatory response driven by LPS and G-CSF. The data help to explain the reduction in markers of organ injury in the ACLF animals treated with TAK-242/G-CSF.

#### Hepatocellular senescence is a feature of ACLF, which is aggravated by G-CSF but prevented by TAK-242

Although modulation of inflammation using TAK-242 abrogated the severity of liver injury in the ACLF animals treated with G-CSF and/or LPS, this data does not uncover the mechanism underlying failure of regeneration in ACLF. Hepatocellular senescence, an essentially state of cell cycle arrest, is a pathophysiological feature of end-stage liver disease that inhibits regeneration and adequate tissue repair, resulting in poor clinical outcomes.<sup>14</sup> Mediators of cell cycle arrest, such as p16 or p21, are expressed in response to tissue injury and transforming growth factor beta-1 (TGFb1) is a key mediator of the senescence-associated secretory phenotype (SASP), where a paracrine spread of injury-independent cellular senescence is observed throughout the liver tissue.<sup>30,31</sup> We therefore evaluated the effect of LPS and interventions with G-CSF and TAK-242 on markers of senescence.

LPS injection in CCl<sub>4</sub>-treated animals led to a prolonged (up to 5 days post administration) upregulation of both p16 mRNA (1.9-

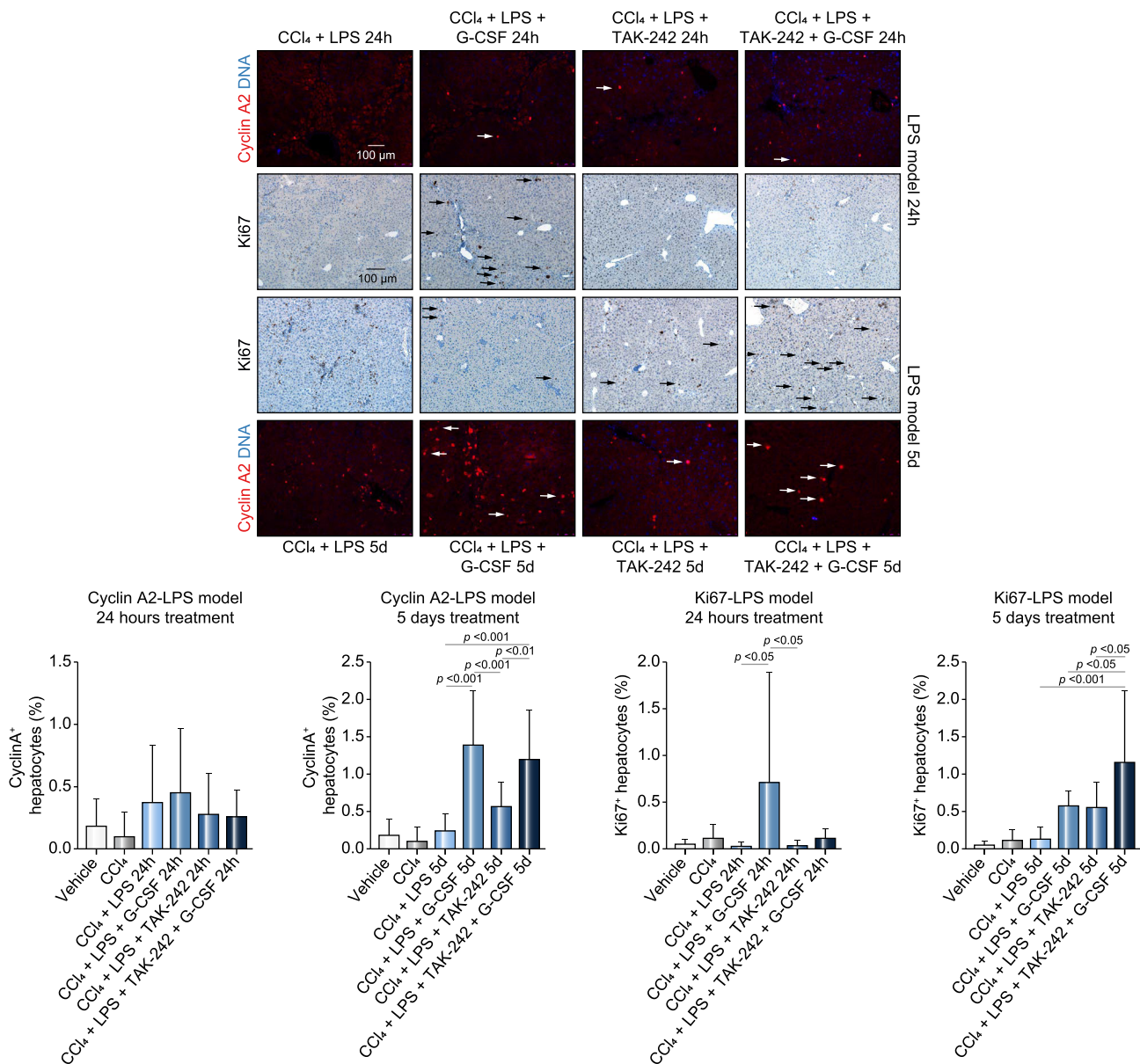
injection led to an increase of CD45<sup>+</sup> circulating haematopoietic cells (n = 4 per group). (C) TAK-242 reduced LPS-driven liver cell death (TUNEL) ( $p < 0.01$ ) (n = 4 per group) and ALT levels (all animals per group). (D) LPS injection increased liver *TNF $\alpha$*  mRNA expression (>2 fold), whereas the additional G-CSF treatment led to an overexpression of *IL-6* mRNA (>2 fold). Expression of both cytokines was reduced after injection of TAK-242 (n = 4 per group). (E) G-CSF treatment after LPS injection led to high numbers of circulating neutrophils and Ly6c-high activated monocytes. TAK-242 reduced the number of both cell subsets and promoted the occurrence of Ly6c-low monocytes in the blood (flow cytometry) (n = 4 per group). (F) TAK-242 decreased the amount of liver-infiltrating F4/80<sup>+</sup> macrophages ( $p < 0.001$ ) and Ly6G<sup>+</sup> neutrophils ( $p < 0.05$ ) (n = 4 per group). Image J was used for image quantification and group comparison was performed by one-way ANOVA and *post hoc* Tukey's multiple comparison only among the following groups: CCl<sub>4</sub>+LPS, CCl<sub>4</sub>+LPS+G-CSF, CCl<sub>4</sub>+LPS+TAK-242, CCl<sub>4</sub>+LPS+G-CSF+TAK-242.  $p > 0.05$  was considered statistically significant. mRNA data is delineated as  $\Delta\Delta$ Ct value. ALT, alanine aminotransferase; CCl<sub>4</sub>, carbon tetrachloride; G-CSF, granulocyte-colony stimulating factor; LPS, lipopolysaccharide. (This figure appears in color on the web.)



**Fig. 3. Evidence of hepatocellular senescence in the rodent model of ACLF and the effect of G-CSF and TAK-242.** (A) Increased hepatic *p16* mRNA expression in CCl<sub>4</sub> + LPS animals with or without treatment with G-CSF treatment, after 24 hours and 5 days. TLR4 inhibition with TAK-242 effectively reduced *p16* mRNA expression at both time points (n = 4 per group). (B) Increased hepatic protein expression of *p21* and TGFb1 in CCl<sub>4</sub>+LPS animals with or without G-CSF treatment, after 24 hours and 5 days. The OD (Western Blot liver lysates) increased from vehicle = 0.67 to CCl<sub>4</sub>+LPS 24 hours = 0.99 and CCl<sub>4</sub> + LPS 5 days = 1.14. Hepatic protein levels of *p21* were raised starting from vehicle = 0.33 to CCl<sub>4</sub>+LPS 24 hours = 0.8 and CCl<sub>4</sub>+LPS 5 days = 1.14. G-CSF exaggerated TGFb1 (OD 1.1) and *p21* expression (OD 0.97) 24 hours after LPS injection. TLR4 inhibition with TAK-242 effectively reduced protein expression of *p21* and TGFb1 at both time points (all individuals per group). Western Blot was repeated twice and a representative blot is shown here. (C) Liver immunohistochemistry demonstrated that *p21* expression occurred predominantly in hepatocytes and could be prevented by TAK-242, with or without G-CSF (n = 3 per group). 24 hours after LPS injection, 2.8% ± 2.2 hepatocytes expressed *p21*, which was enhanced to 8.7% ± 3 by adding G-CSF. This effect diminished 5 days after therapy. TAK-242 added to G-CSF significantly reduced the number of *p21*-expressing (senescent) hepatocytes to 2.0% ± 0.1, 24 hours after LPS injection (p < 0.001). Image J was used for image quantification and group comparison was performed by one-way ANOVA and *post hoc* Tukey's multiple comparison only among the following groups: CCl<sub>4</sub>+LPS, CCl<sub>4</sub>+LPS+G-CSF, CCl<sub>4</sub>+LPS+TAK-242, CCl<sub>4</sub>+LPS+G-CSF+TAK-242. p > 0.05 was considered statistically significant. mRNA data is delineated as  $\Delta\Delta Ct$  value and a greater than 2-fold change in expression was considered as biologically relevant. Western blots were performed with protein lysates pooled from all animals per group and results shown in optical density (OD). ACLF, acute-on-chronic liver failure; CCl<sub>4</sub>, carbon tetrachloride; G-CSF, granulocyte-colony stimulating factor; LPS, lipopolysaccharide; OD, optical density. (This figure appears in color on the web.)

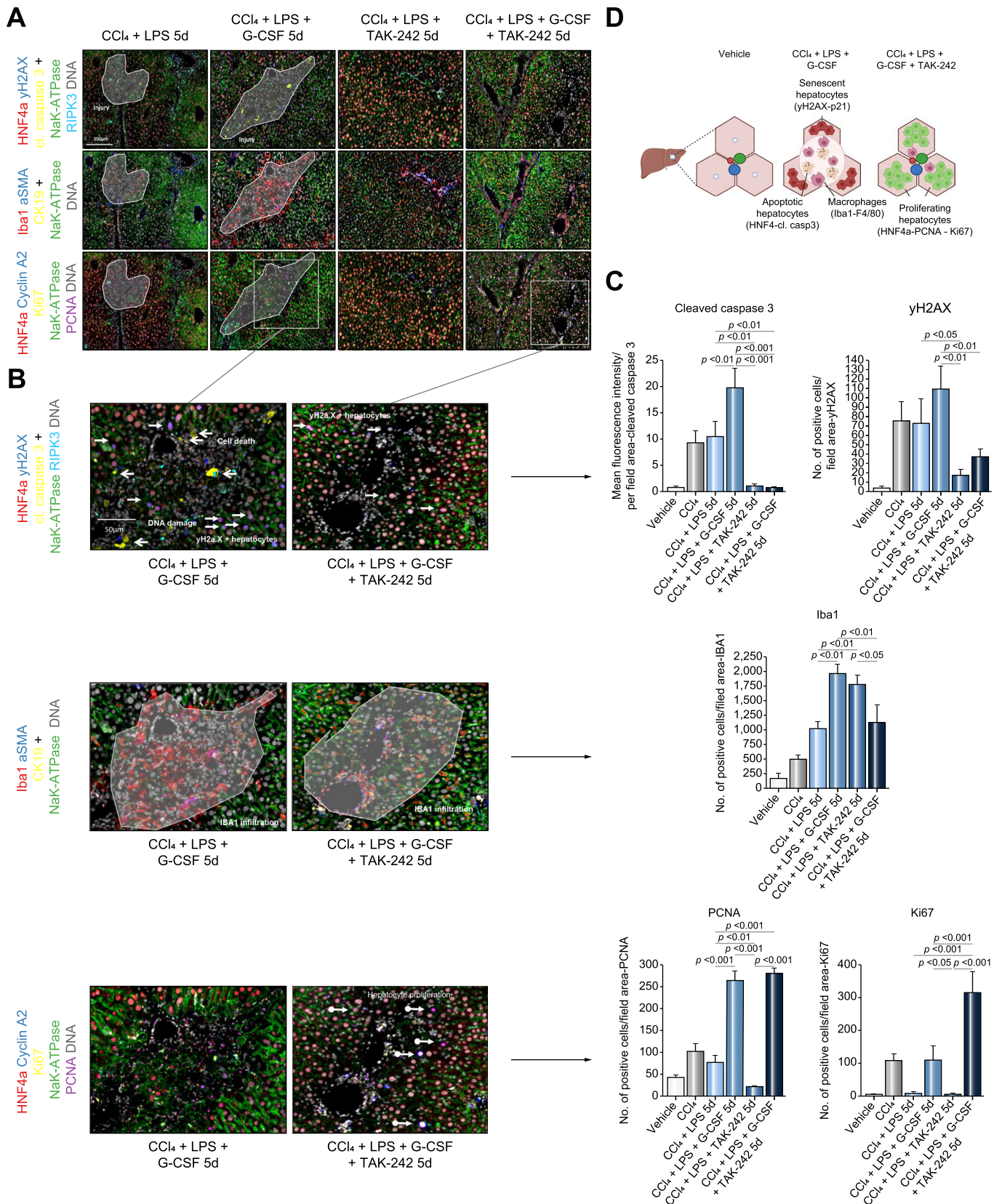
fold compared to vehicles) and TGFb1 in the liver (Western Blot, optical density [OD]: vehicle = 0.67 vs. CCl<sub>4</sub>+LPS 24 hours = 0.99 vs. CCl<sub>4</sub>+LPS 5 days = 1.14). Additionally, hepatic protein levels of p21 (Western Blot: OD vehicle = 0.33 vs. CCl<sub>4</sub>+LPS 24 hours = 0.8 vs. CCl<sub>4</sub>+LPS 5 days = 1.14) and Serpin E1/PAI-1 (proteome profiler) (Fig. 3A/B; Fig. S2C) were also upregulated by LPS, indicating the importance of senescence in this model of ACLF. Liver expression of both markers (p21 and Serpin E1/PAI-1) were further exacerbated by G-CSF treatment (p21 Western Blot OD

CCl<sub>4</sub>+LPS+G-CSF 24 hours = 0.97). The administration of TAK-242, with or without G-CSF, effectively mitigated against the expression of all senescence markers (p16, TGFb1, p21 and Serpin E1/PAI-1) (Fig. 3A/B; Fig. S2C), as shown by mRNA expression (qPCR) and protein expression from pooled liver lysates. p21 immunohistochemistry revealed that activation of the p53/p21 pathway occurred predominantly in hepatocytes. The number of p21-expressing hepatocytes increased after 24 hours from 2.8% ± 2.2 with LPS alone to 8.7% ± 3 with LPS+G-CSF although this



**Fig. 4. Evidence for induction of regeneration markers in hepatocytes induced by TAK-242/G-CSF.** Immunofluorescence and histochemistry staining for Cyclin A2 and Ki67, mediators of cell cycle progression, in paraffin embedded liver tissue (Cyclin A2 upper panel, Ki67 lower panel). In the 24-hour treatment model, G-CSF increased Cyclin A2-expressing hepatocytes (Cyclin A2-positive hepatocytes – CCl<sub>4</sub>+LPS: 0.2% ± 0.2 vs. CCl<sub>4</sub>+LPS+G-CSF: 1.4% ± 0.7). TAK-242 decreased Cyclin A2-positive cells without reaching significance. In the 5-day treatment model, compared to TAK-242 treatment alone, G-CSF in combination with TAK-242 significantly stimulated expression of Cyclin A2 (0.6% ± 0.5 vs. 1.2% ± 0.7; *p* <0.001) and Ki67 (0.6% ± 0.3 vs. 1.2% ± 1; *p* <0.05). Ki67 expression was also enhanced by G-CSF, 24-hrs after LPS injection (*p* <0.05 to CCl<sub>4</sub>+LPS). Image J was used for image quantification and group comparison was performed by one-way ANOVA and post-hoc Tukey's multiple comparison only among the following groups: CCl<sub>4</sub>+LPS, CCl<sub>4</sub>+LPS+G-CSF, CCl<sub>4</sub>+LPS+TAK-242, CCl<sub>4</sub>+LPS+G-CSF+TAK-242. *p* >0.05 was considered statistically significant. CCl<sub>4</sub>, carbon tetrachloride; G-CSF, granulocyte-colony stimulating factor; LPS, lipopolysaccharide. (This figure appears in color on the web.)





**Fig. 5. Multiplex staining of liver tissue depicts localisation of injury, senescence and proliferation after G-CSF and TAK-242 in the CCl<sub>4</sub> – LPS model.** Multiplex immunofluorescence staining on one exemplary sample per group was performed to understand the regional interaction between tissue injury, inflammation and regenerative response. Tissues were sequentially stained for HNF4a (hepatocytes), CK19 (cholangiocytes), Na-K-ATPase (cell membrane), cleaved caspase 3 (cell

effect diminished 5 days after therapy. TAK-242 added to G-CSF significantly reduced the number of p21-expressing (senescent) hepatocytes to  $0.2\% \pm 0.1$ , 24 hours after LPS injection ( $p < 0.001$ ) (Fig. 3C). Therefore, the data suggest that LPS is a key driver of hepatocellular senescence in ACLF, which is further exacerbated by G-CSF. TLR4 inhibition with TAK-242 prevents this senescent phenotype.

#### Failure of regeneration in ACLF is synergistically abrogated by G-CSF and TAK-242

G-CSF has been shown to exhibit regenerative properties in non-inflammatory (not endotoxin-driven) models of liver injury.<sup>32,33</sup> We therefore hypothesised that the excessive inflammation in ACLF, driven by LPS and/or G-CSF as observed above, may prevent G-CSF from acting as pro-regenerative agent and that the addition of TAK-242, which modulates inflammation, may overcome this.

In the CCl<sub>4</sub>-LPS model, therapy with G-CSF for 5 days resulted in hepatocyte activation of Cyclin A2 (Cyclin A2-positive hepatocytes – CCl<sub>4</sub>+LPS  $0.2\% \pm 0.2$  vs. CCl<sub>4</sub>+LPS+G-CSF  $1.4\% \pm 0.7$ ,  $p < 0.001$ ) (Fig. 4), which is known to mediate cell proliferation by promoting cell cycle progression from S- to M-phase; nevertheless, no induction of proliferation was observed, as indicated by the lack of Ki67 expression (Ki67-positive hepatocytes – CCl<sub>4</sub>+LPS  $0.1\% \pm 0.2$  vs. CCl<sub>4</sub>+LPS+G-CSF  $0.6\% \pm 0.2$ , n.s.) (Fig. 4). However, when G-CSF was given for 5 days in combination with TAK-242 (TAK-242+G-CSF), both markers for cell division significantly increased in hepatocytes (Cyclin A2 hepatocyte expression:  $1.2\% \pm 0.7$  [ $p < 0.01$  vs. TAK-242 single treatment]; Ki67 hepatocyte expression  $1.2\% \pm 1$  [ $p < 0.05$  vs. TAK-242 single treatment] (Fig. 4), thus suggesting that TAK-242 enhanced the pro-proliferative effect of G-CSF.

To provide further evidence that G-CSF exhibits a pro-proliferative effect in an environment with lower grade inflammation, we tested 5 days of G-CSF therapy after CCl<sub>4</sub> administration without LPS. In this setting, G-CSF injection significantly increased the number of proliferating hepatocytes; Ki67 staining was observed in  $4.1\% \pm 2.9$  ( $p < 0.001$  to CCl<sub>4</sub>+LPS+G-CSF 5 days) and Cyclin A2 in  $2.1\% \pm 1.3$  ( $p < 0.05$  vs. CCl<sub>4</sub>+LPS+G-CSF 5 days) (Fig. S6).

Protein expression of other regenerative markers was assessed in pooled liver lysates using the proteome array. TAK-242 alone, administered over 24 hours in the CCl<sub>4</sub>-LPS mice, reduced both hepatic markers of vascular regeneration (such as angiopoietin 2, proliferin and platelet-derived growth factor) and other markers involved in liver regeneration, such as IL-22, Flt3-ligand and IGFBP-1 (Fig. S2D&E). Adding G-CSF to TAK-242 markedly increased the liver protein expression of these pro-regenerative markers (Fig. S2D,E).

To provide more details on the link between inflammation, senescence and regeneration, a multiplex immunofluorescence staining of the liver was performed (Fig. 5) including cell type markers (HNF4a – hepatocytes, CK19 – cholangiocytes) as well as

Na-K ATPase for cell membrane delineation. After LPS injection and G-CSF treatment, areas of hepatocyte injury (HNF4<sup>+</sup>) indicated by cleaved caspase 3 positivity ( $p < 0.01$  compared to CCl<sub>4</sub>+LPS) and macrophage infiltration (Iba1<sup>+</sup>) ( $p < 0.01$  compared to CCl<sub>4</sub>+LPS) were surrounded by  $\gamma$ H2AX-expressing hepatocytes, which are associated with senescence. The combination of TAK-242 and G-CSF abrogated liver injury (cleaved caspase 3 –  $p < 0.001$  when comparing CCl<sub>4</sub>+LPS+G-CSF with CCl<sub>4</sub>+LPS+G-CSF+TAK-242) and reduced inflammatory infiltration/response (Iba1 –  $p < 0.01$  comparison between CCl<sub>4</sub>+LPS+G-CSF and CCl<sub>4</sub>+LPS+G-CSF+TAK-242). Therapy with TAK-242 and G-CSF resulted in a decrease of senescent hepatocytes ( $\gamma$ H2Ax –  $p < 0.01$  comparison between CCl<sub>4</sub>+LPS+G-CSF and CCl<sub>4</sub>+LPS+G-CSF+TAK-242) whilst hepatocyte expression of proliferation markers PCNA ( $p < 0.001$  comparison between CCl<sub>4</sub>+LPS and CCl<sub>4</sub>+LPS+G-CSF+TAK-242) and Ki67 ( $p < 0.001$  comparison between CCl<sub>4</sub>+LPS+G-CSF and CCl<sub>4</sub>+LPS+G-CSF+TAK-242) was promoted predominantly in the periportal region (Fig. 5).

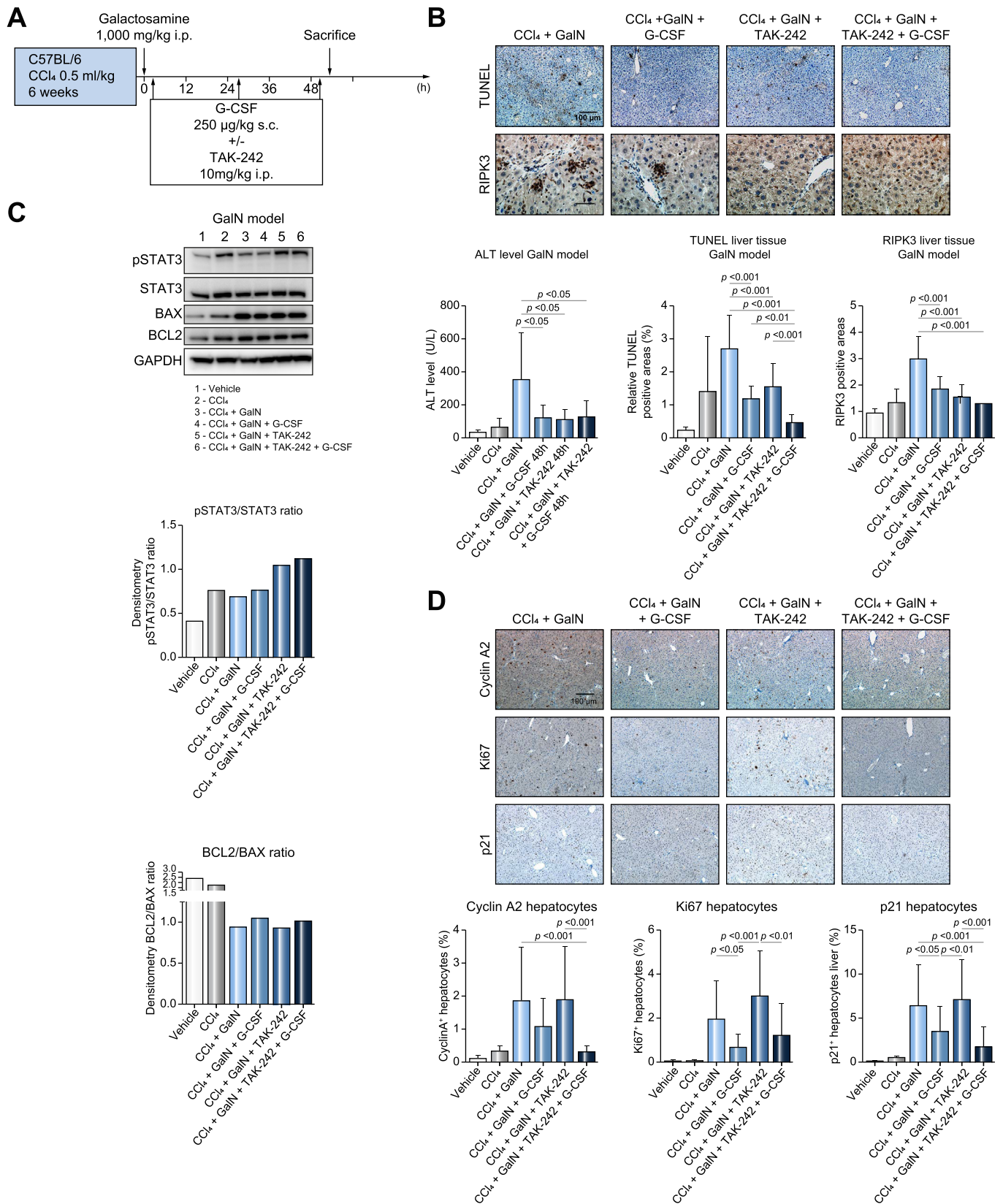
These findings support the hypothesis that G-CSF requires an environment with reduced inflammation to exert its proliferative effects on hepatocytes and, therefore, TAK-242 allows G-CSF to exert its pro-regenerative capacities.

#### TAK-242/G-CSF reduces tissue injury in CCl<sub>4</sub> – GalN-treated mice

The effect of G-CSF, with or without TAK-242, was then tested in a second model where GalN, instead of LPS, was used as a sterile toxic insult in mice treated for 6 weeks with CCl<sub>4</sub> (Fig. 6).

First, we explored the extent of liver injury induced by GalN on the background of CCl<sub>4</sub> and how this was modulated by G-CSF  $\pm$  TAK-242. GalN injection induced a significant liver injury with high ALT levels (27.3 U/L [range 24.2–63.4] vs. 288 U/L [range 46–807]), extended areas of cell death (TUNEL:  $0.23\%$  [range 0.17–0.29] vs.  $2.6\%$  [range 1.3–5.2]), and RIPK3 positivity ( $1\%$  [range 0.75–1.1] vs.  $3.2\%$  [range 1.6–4.4]), a marker for necroptotic cell death. In contrast to the inflammatory LPS model, treatment with G-CSF alone reduced ALT levels (101 U/L [range 51–284],  $p < 0.05$ ), liver cell death (TUNEL  $1.2\%$  [range 0.4–1.9],  $p < 0.001$ ) and RIPK3 expression ( $1.9\%$  [range 0.9–2.5]  $p < 0.001$ ). TAK-242 alone reduced ALT levels (to 96.5 U/L [range 45–229],  $p < 0.05$ ), RIPK3 expression ( $1.7\%$  [range 0.9–2.1],  $p < 0.001$ ) and liver cell death (TUNEL  $1.4\%$  [range 0.4–3.3],  $p < 0.001$ ), however, without reaching statistical significance. The combination of G-CSF and TAK-242 was superior to both single treatments and improved ALT levels (to 74.5 U/L [range 44–297]), liver cell death (TUNEL  $0.45\%$  [range 0.11–0.95],  $p < 0.001$  compared with TAK-242 and  $p < 0.01$  compared with G-CSF) and RIPK3 expression ( $1.23\%$  [range 1–1.78],  $p < 0.001$  compared to CCl<sub>4</sub>+GalN) (Fig. 6B). Furthermore, treatment with TAK-242 + G-CSF was also associated with activation of the STAT3 pathway and a trend to increased anti-apoptotic BCL2 expression as assessed in pooled liver samples by Western Blot (Fig. 6C).

death/injury), RIPK3 (necroptosis),  $\gamma$ H2AX (senescence), Iba1 (macrophage), Cyclin A2 and Ki67 (cell cycle progression). After CCl<sub>4</sub>+LPS+G-CSF there were extended areas of injury with macrophage infiltration and high numbers of senescent and non-proliferating hepatocytes. After abrogation of tissue injury and reduced number of macrophages by TAK-242 and G-CSF there was an increase of proliferating hepatocytes, predominantly in the periportal region. Image processing was performed by Ilastik and CellProfiler was used for quantification. Group comparison was performed by one-way ANOVA and *post hoc* Tukey's multiple comparison only among the following groups: CCl<sub>4</sub>+LPS, CCl<sub>4</sub>+LPS+G-CSF, CCl<sub>4</sub>+LPS+TAK-242, CCl<sub>4</sub>+LPS+G-CSF+TAK-242.  $p > 0.05$  was considered statistically significant. CCl<sub>4</sub>, carbon tetrachloride; G-CSF, granulocyte-colony stimulating factor; LPS, lipopolysaccharide. (This figure appears in color on the web.)



**Fig. 6. Effect of TAK-242/G-CSF in an ACLF model with low-grade inflammation.** (A) C57BL/6 mice were gavaged for 6 weeks with CCl<sub>4</sub> 0.5 mg/ml to induce chronic liver injury (n = 8 per group). Thereafter, GalN was injected to induce a sterile liver injury. G-CSF (250 µg/kg, s.c.) was injected first 1 hour after GalN and continued for 48 hours. TAK-242 was given concurrently (10 mg/kg, i.p.) and continued once daily for 48 hours. (B) GalN injection induced a liver injury with high ALT levels, cell death (TUNEL) and RIPK3 expression (necroptosis). Liver injury was reduced by all treatment options but the combination therapy, TAK-242+G-

We also evaluated whether hepatocyte proliferation was modulated by treatment with G-CSF ± TAK-242 in this model. CCl<sub>4</sub>-treated animals injected with GalN showed high levels of proliferating hepatocytes (Ki67: CCl<sub>4</sub> 0.1% ± 0.1 vs. CCl<sub>4</sub> + GalN 2% ± 1.7; Cyclin A2: CCl<sub>4</sub> 0.1% ± 0.2 vs. CCl<sub>4</sub> + GalN 1.9% ± 1.6) and hepatocytes in cell cycle arrest (p21: CCl<sub>4</sub> 0.5% ± 0.2 vs. CCl<sub>4</sub> + GalN 6.4% ± 4.7). Treatment with G-CSF, with or without TAK-242, reduced the degree of proliferating and senescent hepatocytes, whereas TAK-242 alone did not alter the response to injury (Ki67: CCl<sub>4</sub>+GalN+TAK-242 3% ± 2.1 vs. CCl<sub>4</sub>+GalN+TAK-242+G-CSF 1.2% ± 1.4 [*p* <0.01]; Cyclin A2: CCl<sub>4</sub>+GalN+TAK-242 1.9% ± 1.6 vs. CCl<sub>4</sub>+GalN+TAK-242+G-CSF 0.3% ± 0.2 [*p* <0.001]; p21: CCl<sub>4</sub>+GalN+TAK-242 7.1% ± 4.5 vs. CCl<sub>4</sub>+GalN+TAK-242+G-CSF 1.7% ± 2.3 [*p* <0.001]) (Fig. 6D).

Taken together these data suggest that in this model with low-grade inflammation, the combination of G-CSF and TAK-242 impacts positively on liver injury, regeneration and reduces markers of senescence. It also confirms that the regenerative effect of G-CSF is preserved in an environment with reduced inflammation.

### Relationship between cell death and liver regeneration and the effect of G-CSF and TAK-242

To explore the role of cell death as a modulator and inducer of regeneration, we used RIPA56, a selective RIPK1 inhibitor, to prevent GalN-induced necroptotic cell death in the different groups of animals with CCl<sub>4</sub>-induced chronic liver injury. RIPA56 treatment effectively prevented GalN-driven cell death (total cell death [TUNEL *p* <0.001] and necroptosis [RIPK3 *p* <0.001], both compared to CCl<sub>4</sub>+GalN) and was associated with a decrease in hepatocyte proliferation compared to CCl<sub>4</sub>+GalN (Ki67 2% ± 1.7 vs. 0.2% ± 0.2, *p* <0.001; Cyclin A2 1.9% ± 1.6 vs. 0.03% ± 0.1, *p* <0.001) (Fig. S7), which suggests that in this environment with low-grade inflammation, the regenerative response correlates directly with the severity of liver injury, as previously observed.<sup>30</sup>

We then calculated the ratio between expression of Cyclin A2, as a marker of cell cycle progression, and the severity of total liver cell death (TUNEL) to delineate the pro-regenerative capacity in relation to liver injury of both drugs in all models studied; an increase in the ratio of Cyclin A2/TUNEL would represent enhanced regenerative activity with less cell death and vice versa (Fig. S8). In the 24-hour CCl<sub>4</sub>-LPS model, LPS completely inhibited regenerative responses (CyclinA2/TUNEL ratio: CCl<sub>4</sub> 0.42; CCl<sub>4</sub>+LPS 0.02; CCl<sub>4</sub>+LPS+G-CSF 0.04), with or without G-CSF. In contrast, in the CCl<sub>4</sub>-GalN model the regenerative response was preserved (CyclinA2/TUNEL ratio: CCl<sub>4</sub>+GalN 0.54). In both “short-term” models, administration of TAK-242 was associated with enhanced liver cell regenerative response (CyclinA2/TUNEL ratio: CCl<sub>4</sub>+LPS+TAK-242 1.4; CCl<sub>4</sub>+GalN+TAK-242 0.9) supporting the hypothesis that creating

an ‘inflammation-free’ environment is important to restore regenerative capacities, as also described recently in a model of CCl<sub>4</sub> and *Klebsiella pneumoniae*-induced ACLF.<sup>15</sup> Moreover, the anti-inflammatory environment (in the presence of TAK-242) allowed G-CSF to exert its positive effect on liver injury, notably in the CCl<sub>4</sub>-LPS model, and to enhance regeneration after 5 days of therapy (CyclinA2/TUNEL ratio: CCl<sub>4</sub>+LPS+TAK-242+G-CSF 1.7); thus, supporting the pro-regenerative capacity of G-CSF especially after long-term treatment.

### Discussion

The results of this study provide novel insights into the mechanisms underlying the molecular pathogenesis of ACLF, focusing on the relationship between inflammation and regeneration. The data provide the rationale for combining G-CSF, a recombinant protein that mobilises stem cells from the bone marrow, and a TLR4 inhibitor, TAK-242, that has been shown to reduce hepatic inflammation and mortality in animal models of ACLF. Herein, we show for the first time that in models of LPS- or GalN-induced ACLF, G-CSF and TAK-242 act in combination to improve the severity of liver injury by reducing inflammation and cellular senescence and improving regeneration.

ACLF has a dismal prognosis,<sup>34</sup> and its treatment is an urgent unmet need. In ACLF, TLR4-driven inflammation and lack of hepatic regeneration determines the disease course.<sup>4,12,15,35</sup> Additionally, DAMPs,<sup>36</sup> released from non-apoptotic, immunogenic forms of cell death (such as necroptosis), and PAMPs,<sup>3,4</sup> derived from infection and/or intestinal bacterial translocation, both initiate a cascade of cytokine-driven inflammatory responses that underlie the pathogenesis of the syndrome in which the TLR4 pathway plays an important role.<sup>4,5,8</sup> This systemic inflammation is also associated with the release of endogenous G-CSF, which can induce further tissue injury.<sup>2,35</sup> Paradoxically, G-CSF has been used to treat patients with ACLF, with a view to mobilising the bone marrow stem cell with variable results.<sup>18,19,21</sup> Although preclinical studies suggested that G-CSF may help in tissue repair,<sup>27</sup> and some clinical trials in patients with ACLF showed promising results,<sup>18</sup> the large multicentre randomised clinical trial of G-CSF failed to show a beneficial effect. In fact, in the subgroup of patients with the most severe inflammation, there were some suggestions that G-CSF may even be deleterious.<sup>20</sup>

In a rat model of septic liver injury, 4-day pre-treatment with G-CSF resulted in increased liver expression of LPS-binding protein and TLR4. Administration of LPS to these animals led to enhanced liver cell death and mortality showing a negative sensitising effect of G-CSF.<sup>28</sup> However, in human trials, administration of G-CSF to treat sepsis showed no evidence of efficacy but G-CSF did not induce any excess mortality. It is important to note that these patients had no underlying liver disease.<sup>37</sup> When rats were treated with G-CSF in a rodent model of partial (70%)

CSF, was superior to the single compounds. (C) The treatment effect was associated with activation of the STAT3 pathway and a trend to increased anti-apoptotic BCL2 expression as shown by Western blot in pooled samples. Western blot was repeated twice and a representative blot is shown here. (D) GalN induced a regenerative response with proliferating hepatocytes but also hepatocytes in cell cycle arrest. G-CSF alone or in combination with TAK-242 reduced cell death and the subsequent regenerative response. TAK-242 alone maintained the amount of proliferating (Cyclin A2, Ki67) and senescent (p21) hepatocytes. Image J was used for image quantification and group comparison was performed by one-way ANOVA and *post hoc* Tukey's multiple comparison only among the following groups: CCl<sub>4</sub>+LPS, CCl<sub>4</sub>+LPS+G-CSF, CCl<sub>4</sub>+LPS+TAK-242, CCl<sub>4</sub>+LPS+G-CSF+TAK-242. mRNA data is delineated as ΔΔCt value and a greater than 2-fold change in expression was regarded as biologically significant. Western blots were performed with protein lysates pooled from all animals per group and results shown in optical density (OD). ACLF, acute-on-chronic liver failure; CCl<sub>4</sub>, carbon tetrachloride; GalN, galactosamine; G-CSF, granulocyte-colony stimulating factor; LPS, lipopolysaccharide. (This figure appears in color on the web.)

hepatectomy, administration of LPS was associated with severe liver injury and excess mortality compared with partial hepatectomy and LPS injection alone.<sup>23</sup> These results indicate that G-CSF may sensitise the liver to LPS through TLR4, which may be deleterious in patients with ACLF, as these patients present with high levels of circulating DAMPs and PAMPs, potent ligands for this receptor.<sup>5,9</sup> To better understand the mechanisms underlying the potential deleterious effect of G-CSF, we used murine models of ACLF that mimic the human phenotype. Administration of LPS to mice with advanced fibrosis led to ACLF,<sup>4,25</sup> which was associated with severe inflammation, liver injury, hepatocellular senescence, and high mortality rate. G-CSF exaggerated the inflammatory response and further induced cell death by decreasing the BCL2/BAX ratio and hence favouring cell death. Inhibiting TLR4 receptor signalling with TAK-242, which was associated with marked reduction in the severity of systemic and hepatic inflammation, prevented the increased mortality with G-CSF. Furthermore, TAK-242 allowed G-CSF to act through activation of the STAT3 pathway to prevent further cell death by releasing BCL2 and inducing hepatocyte proliferation. These observations may indicate that the combination may indeed be synergistic in their action rather than simply additive. It may be argued that true recovery needs to be proven at multiple levels, which may be true especially for hepatotoxic injuries in acute liver injury. However, ACLF comprises a complex pathogenesis including inflammation, cell death and inadequate regeneration, with predominance of one or the other in different disease stages. We observed that the combinatorial therapy addresses all aspects and preserves tissue integrity exhibited by low ALT levels and 100% survival. G-CSF may also target side effects of TAK-242, which may impair response to pathogens such as phagocytosis increasing the risk of secondary infections.<sup>38</sup> However, TAK-242 did not increase the risk of infections in human trials and was shown to be safe in sepsis.<sup>39</sup> Nevertheless, any future trial will need to monitor closely for infections when patients are treated with a TLR4 antagonist.

ACLF is characterised by a lack of regeneration and LPS-driven inflammation is potentially of pathogenic importance.<sup>15</sup> In the LPS-precipitated model of ACLF, the number of Ki67<sup>+</sup> hepatocytes were markedly reduced despite activation of Cyclin A2. This may be related to the inhibitory effect of hepatocellular senescence on hepatic regeneration, indicated by increased hepatocellular p21 expression. In human liver tissues of patients with ACLF, the presence of infiltrating CD68<sup>+</sup> macrophages was associated with low numbers of proliferating hepatocytes<sup>6</sup> and in a ACLF mouse model, infection was an important determinant of inhibition of regeneration.<sup>15</sup> Taken together, the data support the hypothesis that targeting inflammation may improve regeneration.

The relationship between TLR4 signalling and cell proliferation is contradictory; previous *in vitro* work on tumour cell lines indicated a pro-proliferative effect on malignant parenchymal cells,<sup>40–42</sup> whereas TLR4 stimulation by LPS was proposed by others to halt pluripotent progenitor cells' ability to divide.<sup>43,44</sup> In cirrhotic animals, administration of LPS inhibited regeneration, as evidenced by activation of the IFN $\gamma$ -STAT1 pathway with increased expression of cyclin-dependent kinase complex inhibitor while reducing the expression of Cyclin D1. Additionally, administration of G-CSF to LPS-induced ACLF animals led to markedly increased expression of p21 in the hepatocytes, which was associated with enhanced expression of other senescence mediators such as Serpin1E, p16 and TGF $\beta$ 1. TLR4 signalling also

leads to TGF $\beta$ 1 release, which is a classical mediator of the senescence-associated secretory phenotype, activating p21 independently of p53.<sup>30,31</sup> Taken together, senescence is an important consequence of LPS-induced ACLF, which is aggravated by G-CSF. These data allow us to hypothesise that senescence and the concomitant inflammatory environment may be involved in the inhibition of hepatic regeneration by senescence in ACLF. The fact that inhibiting the TLR4 pathway by TAK-242 in G-CSF treated animals reduces inflammation, prevents senescence and fosters regeneration further supports this hypothesis. Evidence for the co-existence of pathogen/endotoxin-driven inflammation and lack of regeneration is robust and alcoholic hepatitis, one of the leading precipitating events for ACLF, seems to be one of the potential indications for treatment with this combinatorial therapy. Dubuquoy *et al.* indicated that livers from patients with alcoholic hepatitis lack sufficient hepatocyte proliferation whilst showing increased proliferation of hepatic progenitor cells and ductular reaction.<sup>12</sup> The same disease is also characterised by high levels of circulating LPS and inflammation.<sup>9</sup>

To further validate our observations and determine whether this pro-regenerative effect could be reproduced in an environment of non-LPS-driven, sterile injury, we created a new model of ACLF using GalN on the background of advanced fibrosis. We confirmed similar effects of the combination therapy in this model. Although G-CSF alone reduced tissue injury, its effect on tissue repair, when combining it with TAK-242, was superior to the individual treatments. This observation shows that TAK-242 overcomes the effect of injury-related release of DAMPs and TLR4 activation, confirming the beneficial effect of the combination therapy even when the superimposed injury is non-LPS related.

This study is limited by the fact that ACLF is a heterogeneous entity and animal models might mimic only part of the disease spectrum. However, using an inflammatory and a sterile model of ACLF, mimicking extrahepatic and hepatic insults, allowed us to explore the major pathogenic factors involved in disease development and progression. Although our study provides important insight into the inflammation-senescence-regeneration sequence and provides a potential novel therapeutic strategy, further research is needed to understand the involvement of endothelial cells and other subsets of immune cells (such as macrophages), which might modulate regenerative responses; their involvement is likely as indicated in this study by changes in the markers of vascular regeneration, such as angiopoietin, proliferin and platelet-derived growth factor, possibly also acting through modulation of TGF $\beta$ 1 expression.<sup>45,46</sup> It may also be necessary to apply the combinatorial therapy in models where infection induces ACLF<sup>15</sup> and models of alcoholic hepatitis to test the TAK-242/G-CSF combination in models mimicking the most frequent precipitating events. The models applied here target mechanistic aspects of pathogenetic changes, which occur throughout the whole spectrum of ACLF, providing the basis for translation into humans and for future mechanistic studies.

Although the clinical course of ACLF is not confounded by sex, mechanistic preclinical studies should ideally include both male and female animals. Herein, we chose to use male animals only because the model applied in this study was previously developed in male animals.

In conclusion, the results of this study provide new evidence for the importance of the LPS-TLR4 pathway in modulating the systemic and hepatic inflammation that is associated with ACLF, as well in inducing hepatocyte senescence and inhibiting

regeneration. We report the novel observation that the combinatorial therapy of TLR4 inhibition using TAK-242 and G-CSF reduces liver injury and improves hepatocyte proliferation through reduction in inflammation and senescence. This overcomes the inhibition of hepatic regeneration, a characteristic feature of ACLF. As both drugs are known to be safe, they can be repurposed and evaluated in combination for patients with ACLF.

### Abbreviations

ACLF, acute-on-chronic liver failure; ALT, alanine aminotransferase; BAX, BCL2 associated X protein; BCL2, B-cell lymphoma 2 protein; CCl<sub>4</sub>, carbon tetrachloride; CK19, cytokeratin 19; DAMP, damage-associated molecular pattern; GalN, galactosamine; G-CSF, granulocyte-colony stimulating factor; HMGB1, high mobility group box-1; LPS, lipopolysaccharide; OD, optical density; PAMP, pathogen-associated molecular pattern; PMA, phorbol 12-myristate-13-acetate; SASP, senescence-associated secretory phenotype; TLR4, Toll-like receptor 4; TGFβ1, transforming growth factor beta-1.

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### Conflict of interest

Rajiv Jalan has research collaborations with Yaqrıt. Rajiv Jalan is the founder of Yaqrıt 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 a Founder of Hepyx Ltd. and Cyberliver Ltd. Cornelius Engelmann has received advisory fees from Novartis and CSL Behring. He is shareholder of Hepyx Ltd. Fausto Andreola is shareholder of Hepyx Ltd. Rajiv Jalan, Cornelius Engelmann, Fausto Andreola and Thomas Berg are the named inventors on the patents surrounding the use of G-TAK in ACLF, which have been filed as a priority application. This patent has been licensed to Hepyx Ltd.

Please refer to the accompanying ICMJE disclosure forms for further details.

### Authors' contributions

RJ, TB, FA, CE, FA, ND, SJF - contributed to the conception and design of the study. RJ, ND, FA - provided administrative, study supervision, obtained funding, material support. CE, MF, SFG, FA, AK, SN, LH, MH, AF - 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.

### Data availability statement

Data are available on request from the corresponding author.

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### Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2022.07.006>.

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*Author names in bold designate shared co-first authorship*

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