

Toll-like receptor 5-mediated signaling enhances liver regeneration in mice

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Abstract

Background

Toll-like receptor 5 (TLR5)-mediated pathways play critical roles in regulating hepatic immune response and show hepatoprotective effect in mouse models of hepatic diseases. However, the role of TLR5 in experimental models of liver regeneration has not been reported. This study aims to investigate the role of TLR5 in partial hepatectomy (PHx)-induced liver regeneration.

Methods

We performed 2/3 PHx in wild-type (WT) mice, TLR5 knockout mice, or TLR5 agonist CBLB502 treated mice as a model of liver regeneration. Bacterial flagellin content was measured by ELISA, and hepatic TLR5 expression was determined by quantitative PCR analyses and flow cytometry. To study the effects of TLR5 on hepatocyte proliferation, bromodeoxyuridine (BrdU) incorporation and proliferating cell nuclear antigen (PCNA) expression were analyzed by immunohistochemistry (IHC) staining. The effects of TLR5 during the priming phase of liver regeneration was examined by quantitative PCR analyses of immediate early gene mRNA levels, and western blot analysis of hepatic NF- κ B and STAT3 activation. Cytokines and growth factors production after PHx were detected using real-time PCR analyses and cytometric bead array (CBA) assays. Oil Red O staining and hepatic lipid concentrations were analyzed to examine the effect of TLR5 on hepatic lipid accumulation after PHx.

Results

The bacterial flagellin content in serum and liver was increased and the hepatic TLR5 expression was significantly up-regulated in WT mice upon PHx. TLR5-deficient mice exhibited reduced numbers of BrdU- and PCNA-positive cells, suppressed immediate early gene expression, and decreased cytokines and growth factors production. Moreover, PHx-induced hepatic NF- κ B and STAT3 activation was inhibited in *Tlr5*^{-/-} mice compared with WT mice. Consistently, the administration of CBLB502 significantly promoted PHx-mediated hepatocyte proliferation correlated with enhanced production of proinflammatory cytokines and the recruitment of macrophages and neutrophils in the liver. Besides, *Tlr5*^{-/-} mice displayed significantly decreased hepatic lipid concentrations and Oil Red O positive areas compared to control mice after PHx.

Conclusion

We reveal that TLR5 activation contributes to the initial events of liver regeneration after PHx. Our findings demonstrate that TLR5 signaling positively regulates liver regeneration and suggest a potential application of TLR5 agonist in promoting liver regeneration.

Background

The liver is a unique organ that has a great ability to regenerate itself when a massive loss of hepatic parenchymal cells happens upon hepatic resection [1]. Liver regeneration following partial hepatectomy (PHx) occurs in a multi-step process with at least three important recovery phases: the priming phase, the proliferation phase, and the termination phase [2]. The priming phase is referred to the immediate early phase of liver regeneration, which occurs very rapidly after PHx and lasts for approximately 4 h [2]. The proinflammatory cytokines such as TNF- α and IL-6, which are mainly secreted from nonparenchymal cells, especially Kupffer cells (KCs), act as initiators of the acute phase response. The activation of transcription factors and the increase of the immediate early genes expression such as *c-fos*, *c-jun*, and *c-myc* are hallmarks of this phase [3]. Upon the stimulation of these proinflammatory cytokines, normally quiescent hepatocytes regain proliferative competence and become responsive to hepatic growth factors [4,5,6]. Once hepatocytes pass the restriction point in G1 in the presence of growth factors, the liver irreversibly enters the proliferative phase [2]. However, the underlying mechanisms of PHx-induced activation of transcription factors and the production of cytokines in the liver are still unclear.

Toll-like receptors (TLRs) act as innate immune signal sensors and play central roles in host defense, which are widely expressed on parenchymal and nonparenchymal liver cells [7,8]. In addition to their roles in defense against pathogens, TLRs also significantly contribute to tissue repair and regeneration [9,10,11]. MyD88 is a common adaptor protein required for TLRs-mediated signaling pathways activation and proinflammatory cytokine production [12,13]. MyD88 deletion impairs liver regeneration by attenuating the activation of NF- κ B and decreasing the expression of immediately early genes and the production of TNF- α and IL-6 in KCs after PHx, which highlights that TLRs/MyD88 signaling pathways are crucial for PHx-induced liver regeneration [14,15]. However, further studies have excluded the possible contribution of TLR2, TLR4, or TLR9 to MyD88-mediated pathways in liver regeneration after PHx in mice [15]. In addition, TLR3 signaling which uses a distinct adaptor protein TRIF but not MyD88 was shown to attenuate the initiation of liver regeneration [16]. Therefore, liver regeneration is likely to be driven by some other TLRs via the MyD88 signaling pathway.

TLR5 serves as the main receptor for bacterial flagellin and plays a critical role in regulating the response to ionizing radiation [17,18] and immune response [19,20,21]. CBLB502, a pharmacologically optimized flagellin derivative, has been reported to protect mice from lethal total-body irradiation-induced gastrointestinal and hematopoietic acute radiation syndromes [17] and ionizing radiation-induced male reproductive system damage [18]. In recent years, the liver has been identified as one of the most sensitive organs to CBLB502. Administration of CBLB502 strongly activates NF- κ B-, STAT3-, AP1-, and PREM- driven pathways in the liver, resulting in the production of many immunomodulatory factors and massive infiltration of immune cells [22]. Moreover, TLR5-mediated pathways were shown to protect mice from anti-Fas antibody- or concanavalin A-induced fulminant liver injury by limiting hepatocytes apoptosis or T/NKT cell activity [22,23]. Besides, our own and others' studies have indicated that TLR5 signaling serves as a regulator in hepatic immune cell activation and cytokine production [22,23,24,25]. Importantly, injection of CBLB502 alone in wild-type (WT) mice induced a rapid increase of serum or hepatic TNF- α , IL-6, and G-CSF, all of which are shown to contribute to liver regeneration [22,26]. Together, these data suggest that TLR5-mediated pathways may be involved in the regulation of liver regeneration.

To the best of our knowledge, the effect of TLR5-mediated pathways on the experimental models of liver regeneration has not been studied previously. In this paper, we aimed to determine the role of TLR5 in PHx-induced liver regeneration.

Methods

Animals

Tlr5^{-/-} mice on a C57BL/6 background from Jackson Laboratory were kindly provided by Prof. Huimin Yan (Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China). Specific pathogen-free (SPF) C57BL/6 mice were purchased from Vital River Experimental Animal Company (Beijing, China). Male mice between 8 and 10 weeks were used in the study. In all experiments, genetically modified mice were systematically compared to their age- and weight-matched WT littermates. All animals were maintained in a temperature-controlled, specific pathogen-free room with 12-hour light and dark cycle and *ad libitum* diet (standard laboratory chow and water) in the Experimental Animal Center of the Academy of Military Medical Sciences according to the National Laboratory Animal guidelines (Ministry of Health, P.R. China, 1998).

Partial Hepatectomy Model

For 2/3 partial hepatectomy studies, male mice between 8 and 10 weeks were anesthetized using 1% pentobarbital sodium. A midline laparotomy was performed by aseptic removal of the central and left lobes [27]. Animals of the control group underwent midventral laparotomy without manipulation of the liver (sham surgery). Age- and weight-matched *Tlr5*^{-/-} and WT littermates were treated with gentamycin for 1 week before 2/3 partial hepatectomy or sham surgery to deplete gram-negative, gut-derived bacteria [23]. Experiments started between 8:00 AM and 12:00 PM. For CBLB502 administration, mice were pretreated with a single dose of CBLB502 (0.2 mg/kg) intraperitoneally 1 hour before partial hepatectomy.

Gene expression datasets

For gene expression analysis, a published dataset was downloaded from the GEO repository (GSE95135). Raw data were normalized by the robust multiarray average (RMA) method. We use ExpressVis (<http://www.fgvis.com/expressvis/>) to visualize the expression of specific genes of interest [28].

Flow cytometry

Liver mononuclear cells (MNCs) were obtained as previously described [23]. Briefly, the liver tissue was dissected, and dissociated in collagenase (Sigma-Aldrich, C5138) digestion. Liver specimens were pressed through a 40 µm strainer. The single-cell suspension was centrifuged by 50 *g* for 5 min, and the supernatant was further centrifuged at 320 *g* for 5 minutes. The pellet containing liver mononuclear cells was resuspended in 10 ml of 30% Percoll (GE, 17089102), then centrifuged at 800 *g* for 15 minutes

without brake. The cell pellet was washed and then treated with red blood cells lysis solution (TIANGEN Biotech, RT122). Centrifuge at 400 *g* for 10 min, resuspend the mononuclear cell pellets in RPMI-1640 medium for Flow cytometric analysis. MNCs were stained with F4/80-FITC, CD45.2-PE-Cy7, Siglec F-APC, Ly6G-eFluor 450, and CD11b-BV605 to identify liver neutrophils, KCs, and recruited macrophages. TLR5-PE or IgG2a-PE antibody was further stained to detect the mean fluorescence intensity (MFI) of TLR5 expression on these cells. After gating the indicated cells, the Mean of TLR5-PE in the statistics window was used to calculate the TLR5 MFI. The antibodies used were shown in Table S2. The number of MNCs was counted using counting beads. Flow cytometric analysis was performed using a BD FACSCalibur instrument and FlowJo software.

Histological analysis, Oil Red O, and immunohistochemistry stainings

Liver tissues were excised. Fixed tissues in paraformaldehyde and then embedded tissues in paraffin. H&E was performed for morphological analysis. Liver tissues were frozen directly in OCT compound for Oil Red O staining. The liver regeneration rate was determined by PCNA immunohistochemistry staining and the incorporation of bromodeoxyuridine (BrdU). For BrdU incorporation, mice were injected 85 µg/kg BrdU and sacrificed 2 hours later, and incorporation was visualized immunohistochemically using BrdU immunohistochemistry staining. BrdU and PCNA immunohistochemistry staining were performed following standard protocols (Wuhan Servicebio technology co., LTD), and the percentage of positive cells was analyzed from randomly selected 3 fields of ×200 magnification for each sample. Oil Red O positive areas were quantified in 3 fields per slide under light microscopy (×200). All pictures of the liver sections were captured by Nikon Digital Sight DS-U3 camera. Scale bar, 50µm. Image analysis procedures were performed with IPP v6.0.

Measurements of Cytokines, flagellin concentrations, and aminotransferases

To detect the cytokines in serum, Mouse TNF Flex Set, Mouse IL-6 Flex Set, Mouse G-CSF Flex Set, Mouse HGF ELISA kit (Abcam, ab223862), Mouse TGF-α ELISA kit (Cloud-Clone Corp., SEA123Mu) were used according to manufacturer's instruction. We used a mouse flagellin ELISA kit (Beijing chengzhikewei biotechnology Co., SU-BN28100) for the quantitative determination of serum and liver homogenates flagellin concentrations. Serum ALT and AST were measured according to the IFCC primary reference procedures at Beijing CIC Clinical Laboratory (Beijing, China).

Detection of lipids

Serum lipids quantification was performed with Cholesterol kit (Wako, 294-65801), Triglyceride kit (Wako, 290-63701), and NEFA kit (Wako, 294-63601) following standard methods. Hepatic lipids were assayed by Cholesterol kit, Triglyceride kit, and Free fatty acids assay kit.

Quantitative PCR

Hepatic RNA was extracted by TRIzol and reverse-transcribed into cDNAs. The cDNAs were amplified with Quantitative PCR Mix. Relative gene expression was evaluated by the ΔCT method, and *Actb* was used as

an internal control. Genes specific primers were designed by Primer Bank and listed in Table S1 in Supplementary Material.

Western blot

Proteins were extracted from liver specimens homogenized with PBS containing proteinase inhibitor cocktail (one tablet for 50 ml, Roche, 04693116001) and 0.5% Triton X100. Liver total protein quantification was performed with Protein Quantification Assay Kit. Protein extracts were denatured in Laemmli buffer and then separated using SDS PAGE. Proteins were diverted from the gel onto the PVDF membrane and then probed with indicated primary antibodies. Immune complexes on the membrane were detected with HRP conjugated secondary antibodies. The antibodies used were shown in Table S2.

Statistical analysis. Statistical data were calculated with GraphPad Prism 7. Continuous variables are presented as mean \pm standard deviation (SD). Data from two or three independent experiments with similar results were expressed as means \pm standard error of the mean (SEM). A standard two-tailed unpaired Student's *t*-test was used to test the significance of differences between the two groups. The distribution of variables is tested by the Kolmogorov-Smirnov test. *P*-value <0.05 was considered statistically significant. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

Results

3.1 TLR5 is up-regulated in the liver after PHx

We used 2/3 PHx as an established model of liver regeneration to determine the effect of TLR5 [27]. Significant increases of bacterial flagellin in the serum and liver were observed at 6 hours post PHx (Fig. 1a). We next investigated the expression pattern of TLR5 in the liver of mice after PHx. Global transcriptome analysis of the mouse liver at various time points following PHx revealed a significant up-regulation of TLR5 along with other TLRs (GSE95135) [29] (Fig. 1b). Consistently, real-time PCR analysis showed that the expression of TLR5 in the liver was significantly increased following PHx, with two peaks occurring at 1 hour and 12 hours after PHx (Fig. 1c). By flow cytometry, we found that TLR5 was expressed on hepatic neutrophils, KCs, and recruited macrophages (Fig. 1d-e), which is line with the previous results of our own and others [8,23]. The mean fluorescence intensity of TLR5 on these cells was unchanged, but the numbers of neutrophils and recruited macrophages were significantly increased following PHx, which might account for the up-regulation of hepatic TLR5 expression in the early phase upon PHx (Fig. 1f).

3.2 TLR5 deficiency decreases hepatocyte proliferation after PHx

To assess the effect of TLR5 on liver regeneration, we performed 2/3 PHx in *Tlr5*^{-/-} mice and their WT littermates. The hepatocyte proliferation, as assessed by the incorporation of BrdU, was remarkably

decreased in *Tlr5*^{-/-} mice at 36 and 48 hours after PHx (Fig. 2a-b). Consistent with this result, PCNA staining showed a reduced number of proliferating hepatocytes in *Tlr5*^{-/-} mice at 36 hours after PHx (Fig. 2c-d). The serum ALT and AST levels were rapidly elevated in mice after PHx, but no significant difference was observed between WT and *Tlr5*^{-/-} mice (Fig. 2e), indicating a similar degree of liver injury in the two genotypes. Together, these data suggest that deficiency of TLR5 significantly decreases hepatocyte proliferation in the first 48 hours after PHx.

3.3 TLR5 deficiency suppresses hepatocyte priming in PHx-induced liver regeneration

As flagellin concentration and TLR5 expression in the liver increased at early time points after PHx, we examined the effects of TLR5 on the priming of liver regeneration following PHx. Quantitative PCR was used to examine gene expressions involved in the initial stage of liver regeneration. The results showed that PHx increased the hepatic mRNA levels of c-Myc, c-Jun, and c-Fos in both *Tlr5*^{-/-} and WT mice at 30 to 60 min following PHx, but the increase was significantly blunted in *Tlr5*^{-/-} mice (Fig. 3a), suggesting that loss of TLR5 suppresses PHx-induced immediate early gene expression.

We further investigated the effect of TLR5 signaling on proinflammatory cytokines expression upon PHx. As shown in Fig. 3b, serum levels of TNF- α , IL-6, TGF- α , and HGF in *Tlr5*^{-/-} mice were similar to those in WT mice before PHx. PHx rapidly increased the serum levels of these cytokines in both WT and *Tlr5*^{-/-} mice; however, these effects were greater in WT mice at 6 and 12 hours after PHx (Fig. 3b). Consistently, TNF- α , IL-6, TGF- α , and HGF mRNA levels in the liver were inhibited in *Tlr5*^{-/-} mice at 1 and 3 hours after PHx (Fig. 3c). These results indicate that TLR5 signaling contributes to cytokines production induced by PHx.

Activation of NF- κ B and STAT3 are well known as one of the major priming events during liver regeneration [3,14]. NF- κ B signaling is activated in mouse liver by PHx as indicated by the phosphorylation and degradation of I κ B α , and the p65 phosphorylation. Compared with WT mice, PHx-induced NF- κ B activation in the liver was inhibited in *Tlr5*^{-/-} mice at 30 and 60 min after PHx (Fig. 3d). Both WT and *Tlr5*^{-/-} mice displayed increased hepatic STAT3 phosphorylation levels at 30 and 60 min following PHx, while *Tlr5*^{-/-} mice showed lower levels of phosphorylated STAT3 than WT mice (Fig. 3d). Our results indicate that TLR5 contributes to the regulation of hepatocyte priming in liver regeneration after PHx.

3.4 The TLR5 agonist CBLB502 enhances hepatocyte proliferation in mice following PHx

To further assess the role of the TLR5 pathway in liver regeneration, we investigated whether administration of CBLB502, a TLR5 agonist derived from *Salmonella* flagellin, affects liver regeneration in mice after PHx. The liver/body weight ratio of CBLB502-pretreated mice was significantly higher than that of control mice during the first 72 hours post-PHx, suggesting that activation of the TLR5 pathway is involved in the early recovery of liver mass after PHx (Fig. 4a). Accordingly, PHx-induced liver damage was reduced by CBLB502 administration at 24 and 72 hours after PHx as revealed by decreased serum transaminases (Fig. 4b). Hepatocyte proliferation was markedly enhanced in CBLB502 treated mice at 36, 48, and 72 hours after hepatectomy by BrdU staining and 36 and 48 hours by PCNA staining (Fig. 4c-f). Taken together, activation of TLR5 signaling by CBLB502 enhances hepatocyte proliferation in mice following PHx.

3.5 Enhanced inflammatory response in CBLB502-pretreated mice after PHx

In line with previous reports [23], the administration of CBLB502 in mice rapidly increased serum TNF- α , IL-6, and G-CSF (Fig. 5a). Interestingly, TGF- α and HGF, which are critical for liver regeneration [30,31], were also significantly up-regulated in the serum at 3 and 6 hours after CBLB502 injection (Fig. 5b). We next performed PHx 1 hour after CBLB502 administration. Much higher levels of serum TNF- α , IL-6, G-CSF, TGF- α , and HGF were observed in CBLB502-pretreated mice than in control mice right before PHx (Fig. 5c-d). After PHx, the serum concentrations of these growth factors were significantly increased in control mice, but the levels were still much lower than those in CBLB502-pretreated mice at 6 hours after PHx (Fig. 5c-d). Moreover, the administration of CBLB502 significantly increased hepatic mRNA levels of c-Fos, c-Myc, c-Jun, TNF- α , and IL-6 at 1 hour post-PHx (Fig. 5e), which is in agreement with the result that TLR5 deficiency suppresses PHx-induced immediate early gene expression. We next examined whether CBLB502 affects the hepatic recruitment of immune cells after PHx. As shown in Fig. 5f, the number of hepatic mononuclear cells (MNCs), neutrophils, and recruited macrophages was significantly higher in CBLB502 treated mice than that in control mice before PHx, which was in line with previous reports that CBLB502 treatment induces recruitment of various types of immune cells into the liver [22,23]. PHx also induced a significant increase in the total number of hepatic MNCs, as well as the number of recruited macrophages and neutrophils, and mice pretreated with CBLB502 showed further increased number of these cells compared to mice pretreated with PBS. However, the number of KCs was not affected (Fig. 5f). Taken together, these data indicate that CBLB502 pretreatment increases the hepatic inflammatory response in mice after PHx.

3.6 TLR5 signaling contributes to hepatic lipid accumulation induced by PHx

The liver transiently accumulates lipids during liver regeneration, which is associated with hepatocyte proliferation [32,33,34]. Histological analysis and Oil Red O staining showed an obvious decrease of

hepatic lipid accumulation in *Tlr5*^{-/-} mice at 24 hours post-PHx (Fig. 6a). The levels of triglyceride, free fatty acids, and cholesterol in liver homogenates were comparable between WT and *Tlr5*^{-/-} mice under normal conditions (Fig. 6b). Significant increases of triglyceride and free fatty acids levels in liver homogenates were observed in both WT and *Tlr5*^{-/-} mice at 24 hours after PHx, but the levels of triglyceride and free fatty acids in *Tlr5*^{-/-} mice were remarkably lower than those in WT mice (Fig. 6b). Deficiency of TLR5 did not affect the levels of hepatic cholesterol and serum triglyceride, free fatty acids, and cholesterol no matter before or after PHx (Fig. 6b-c). Consistent with these results, CBLB502 treatment increased hepatic triglyceride and free fatty acids accumulation at 24 and 36 hours following PHx, but had no significant effect on hepatic cholesterol content (Fig. 6d). Histological examination and Oil Red O staining of liver sections from CBLB502-treated and WT mice at different time points after PHx further confirmed these results (Fig. 6e). Collectively, these data indicate that the TLR5 signaling pathway is involved in the regulation of the hepatic lipid transient accumulation induced by PHx.

Discussion

Although previous studies have shown that activation of TLR5 signaling has a significant effect on the immune-privileged status of the liver and protects against ConA and Fas-agonistic antibodies-induced liver injury [19,20,21,22,23], the role of TLR5 in liver regeneration has not been reported. In this study, we have identified a role for TLR5 in liver regeneration using TLR5 knockout mice and TLR5 agonist CBLB502 treated mice. We provided several lines of evidence suggesting that the activation of TLR5 signaling positively regulates liver regeneration via enhancing proinflammatory responses in the liver. First, bacterial flagellin content was increased in serum and liver after PHx. Meanwhile, the expression of TLR5 in the liver was significantly up-regulated. Second, loss of TLR5 resulted in inhibition of PHx-induced liver regeneration, which is associated with attenuation of NF-κB and STAT3 activation, proinflammatory cytokines production, and immediate early gene expression. Third, activation of TLR5 signaling by TLR5 agonist CBLB502 significantly promoted PHx-mediated hepatocyte proliferation, which is accompanied by enhanced production of proinflammatory cytokines and recruitment of macrophages and neutrophils in the liver. Microbiota composition alteration is associated with elevated fecal bioactive flagellin and LPS levels, which may affect liver regeneration [35,36,37,38,39]. In the present study, we treated mice with gentamycin as previously described, which results in comparable microbial composition in the intestinal tract between *Tlr5*^{-/-} and WT mice [23], thereby eliminates the effects of pathogenic intestinal bacteria on PHx-induced liver regeneration in *Tlr5*^{-/-} mice. To our knowledge, the present study is the first report that TLR5 is required for liver regeneration after PHx. CBLB502 is a pharmacologically optimized *Salmonella* flagellin derivative that is less immunogenic than wild-type flagellin but retains the activity to promote NF-κB activation via TLR5, and it is now under development as an antitumor drug and medical countermeasure for radiation [17,22,24]. Our own studies have shown that CBLB502-mediated protective effects against concanavalin A-induced hepatitis and male reproductive system damage induced by ionizing radiation mainly rely on the TLR5 pathway [18,23], which was in line with the previous report that radioprotection by CBLB502 is indeed TLR5-dependent [17]. Thus, our

findings suggest that activation of TLR5 signaling may have potential for the improvement of liver regeneration when the liver is compromised.

It is well known that several cytokines such as TNF- α and IL-6 produced by nonparenchymal liver cells and transcription factors such as NF- κ B and STAT3 are crucial for liver regeneration. After PHx, TLRs/MyD88-mediated pathways activate NF- κ B pathways in nonparenchymal liver cells, induce TNF- α and IL-6 production, and trigger immediate early gene expression in hepatocytes [3,4,14]. In TLR5 knockout mice, initiation of liver regeneration is abated at the earlier time point as evidence by suppression of NF- κ B and STAT3 activation, IL-6 and TNF- α production, and immediate early gene expression after PHx, suggesting that TLR5 signaling is required for liver regeneration, especially for the priming phase. This conclusion is supported by the fact that CBLB502 treatment enhanced hepatic c-Myc, c-Fos, c-Jun, IL-6, and TNF- α expression in mice in the early period following PHx. Although Burdelya *et al* reported that hepatocytes were the effector cell specifically and directly responding to CBLB502 [22], previous studies showed a very low level of TLR5 expression on hepatocytes, as well as a fairly weak response of hepatocytes to CBLB502 *in vivo* [23,40], which indicate TLR5 signaling may regulate PHx-induced liver regeneration through nonparenchymal liver cells. It is now almost universally accepted that NF- κ B activation in KCs is crucial for intact liver regeneration after PHx [3,4,41]. Importantly, KCs were shown to express TLR5 in our present study, which is consistent with previous works [8]. Although the number of KCs in the liver and the expression of TLR5 on KCs were unaltered after PHx, the levels of bacterial flagellin in serum and liver were increased in PHx-treated mice. Thus, the activation of TLR5 signaling in KCs may be enhanced following PHx and contribute to liver regeneration. In addition, hepatic recruited macrophages and neutrophils, which were shown to express TLR5 [8,23], have been demonstrated to accelerate liver regeneration via the TNF/FasL/Fas or STAT3 pathway [42,43]. After PHx, the expression of TLR5 on recruited macrophages and neutrophils was not altered, but their number was significantly increased, suggesting that the up-regulation of hepatic TLR5 expression induced by PHx may be attributed to the influx of TLR5-positive immune cells. Studies of our own and others showed that administration of CBLB502 rapidly induced the expression of numerous immunomodulatory factors including TNF- α and IL-6 and massive infiltration of many kinds of immune cells such as macrophages and neutrophils in the liver of mice without PHx [22,23], and the extent was further enhanced in the case of PHx. Therefore, it is likely that the early and transient activation of hepatic TLR5 after PHx increases inflammation response during liver regeneration, by which TLR5 signaling promotes liver regeneration.

Except for TNF- α and IL-6, TLR5 signaling also affected TGF- α , HGF, and G-CSF expression after PHx. A marked decrease in systemic and liver local TGF- α , HGF, and G-CSF was detected in TLR5 knockout mice after PHx. In contrast, CBLB502 pretreatment significantly increased their expression. TGF- α is an autocrine stimulator of hepatocyte proliferation [31,44]; HGF is produced in the liver by nonparenchymal cells and acts as a complete mitogen to promote hepatocyte proliferation through a paracrine mechanism [30,45]. Their important roles in liver regeneration have been extensively reported [1,2,4,44,45]. These findings indicate that the TLR5 pathway also regulates the proliferation phase of liver regeneration through the induction of hepatocyte proliferation-associated growth factors. The induction of G-CSF by CBLB502 plays an important role in the drug's ability to protect mice against radiation injury [17].

Previous studies also showed that G-CSF facilitates liver regeneration by suppression of hepatic NK cells and increasing the migration of BM-derived progenitors to the liver [46,47]. Our previous studies showed that TLR5 signaling can restrain T/NKT cell activation in the liver [23]. Thus, TLR5 signaling-induced facilitation of liver regeneration may also be achieved by immunoregulation of NK or/and NKT cells. Transient hepatic lipid accumulation during liver regeneration is essential for hepatocyte proliferation induced by PHx [32,33,34,48]. Our results showed that TLR5 signaling significantly increases the levels of triglyceride and free fatty acids in the early regenerating liver, indicating that TLR5 signaling may contribute to lipid accumulation during early liver regeneration. These findings are worth further studies in the future.

There are several limitations to this study. First, the analysis of the effect of the TLR5 pathway in liver regeneration was focus on the priming phase and the proliferation phase after PHx, but it was unclear whether the TLR5 pathway would affect the termination phase in liver regeneration. Second, as the study was conducted in mice on a C57BL/6 background, more research is needed to confirm these results in other animal species, particularly the primates. Additionally, our data indicate that TLR5 signaling contributes to hepatic lipid accumulation induced by PHx, however, the mechanism still needs further study. Finally, our observation should be further verified with clinical samples before extrapolating to human beings.

Conclusions

This work provides some evidence that TLR5 activation contributes to the initial events of liver regeneration following PHx. Our findings open a new door for a better comprehension of the mechanisms of liver regeneration and suggest a potential application of TLR5 agonist in promoting liver regeneration.

Declarations

Availability of data and material

All materials are commercially available, and the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Contributions

WZ conducted the experiments. LW, XHS, XL, YX, JZ, TW, YTL collected and analyzed the data. HC, YQZ, MY, CHG, CYL gave technical or material support. GMR contributed to the study design. WZ, RHY, and XMY drafted the manuscript. XMY and RHY supervised the project. LW and MY obtained funding. All authors read and approved the final manuscript.

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Ethics declarations

Ethics approval and consent to participate

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Beijing Institute of Lifeomics (NO. IACUC-DWZX-2020-568).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abbreviations

ALT: Alanine transaminase; AST: Aspartate transaminase; BrdU: Bromodeoxyuridine; CBA: Cytometric bead array; Con A: Concanavalin A; H&E: Hematoxylin and eosin; IHC: Immunohistochemistry; IL-6: Interleukin-6; KCs: Kupffer cells; MNCs: Mononuclear cells; MyD88: Myeloid differentiation factor 88; PCNA: Proliferating cell nuclear antigen; PHx: Partial hepatectomy; SEM: Standard error of the mean; SPF: Specific pathogen-free; TLR5: Toll-like receptor 5; TLRs: Toll-like receptors; TNF- α : Tumor necrosis factor- α ; WT: Wild-type

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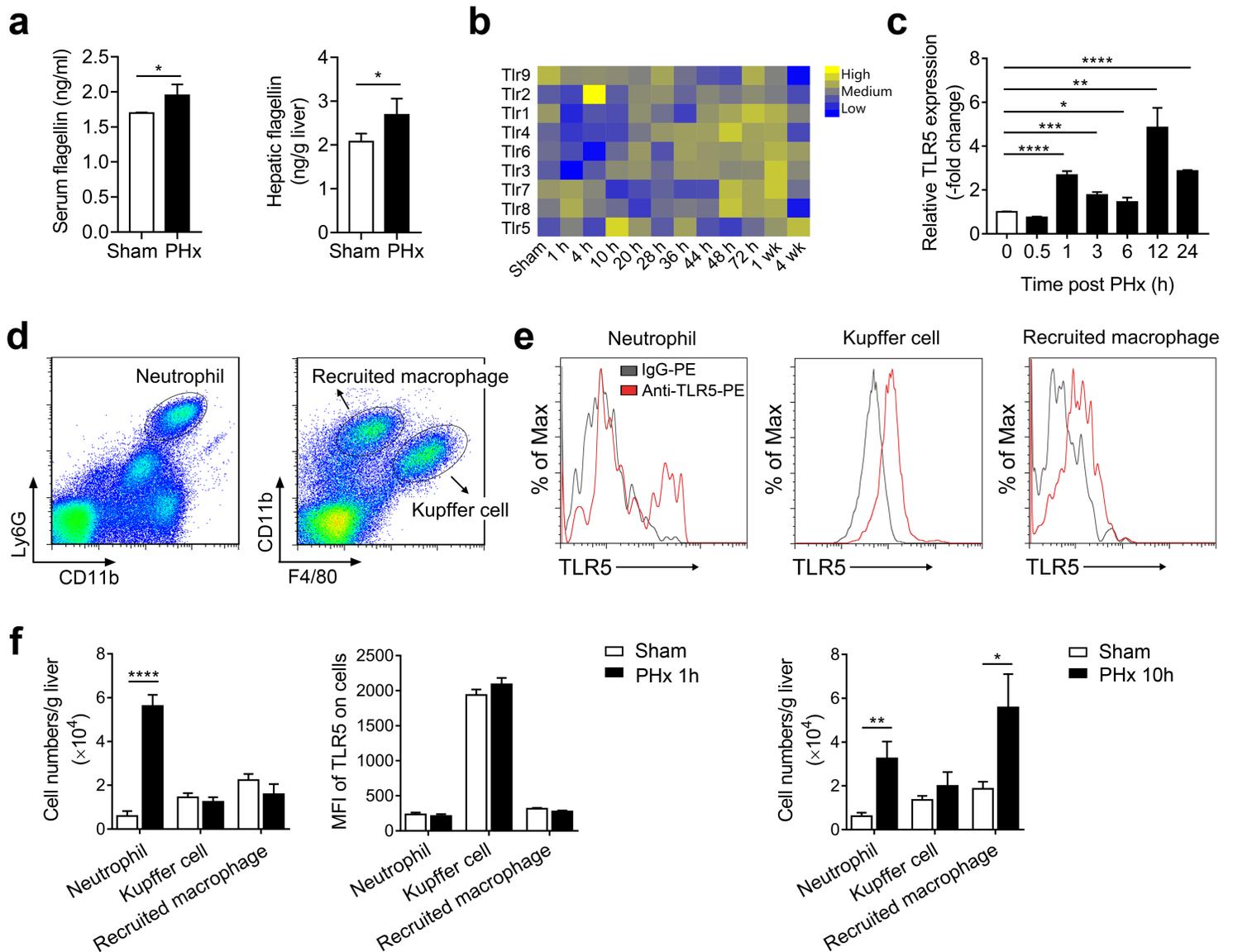
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Figures

Figure 1**Figure 1**

TLR5 is up-regulated in the liver after PHx. a. Flagellin concentrations in serum and liver of WT mice 6 hours following sham surgery or PHx. b. TLRs expression levels in regenerating WT mice liver upon PHx from published transcriptome dataset (GSE95135). c. Relative mRNA levels of hepatic TLR5 at the indicated times following PHx. Flow cytometry gating strategy (d) and TLR5 expression levels (e) of liver neutrophils, KCs, and recruited macrophages in WT mice. f. Cell counts and mean fluorescence intensity of TLR5 on liver neutrophils, KCs, and recruited macrophages at the indicated times upon PHx. Panel a

show means \pm SD. Data are representative of three independent experiments. Panel c and f show means \pm SEM. Data are derived from two or three independent experiments with similar results. n = 3-6.

Figure 2

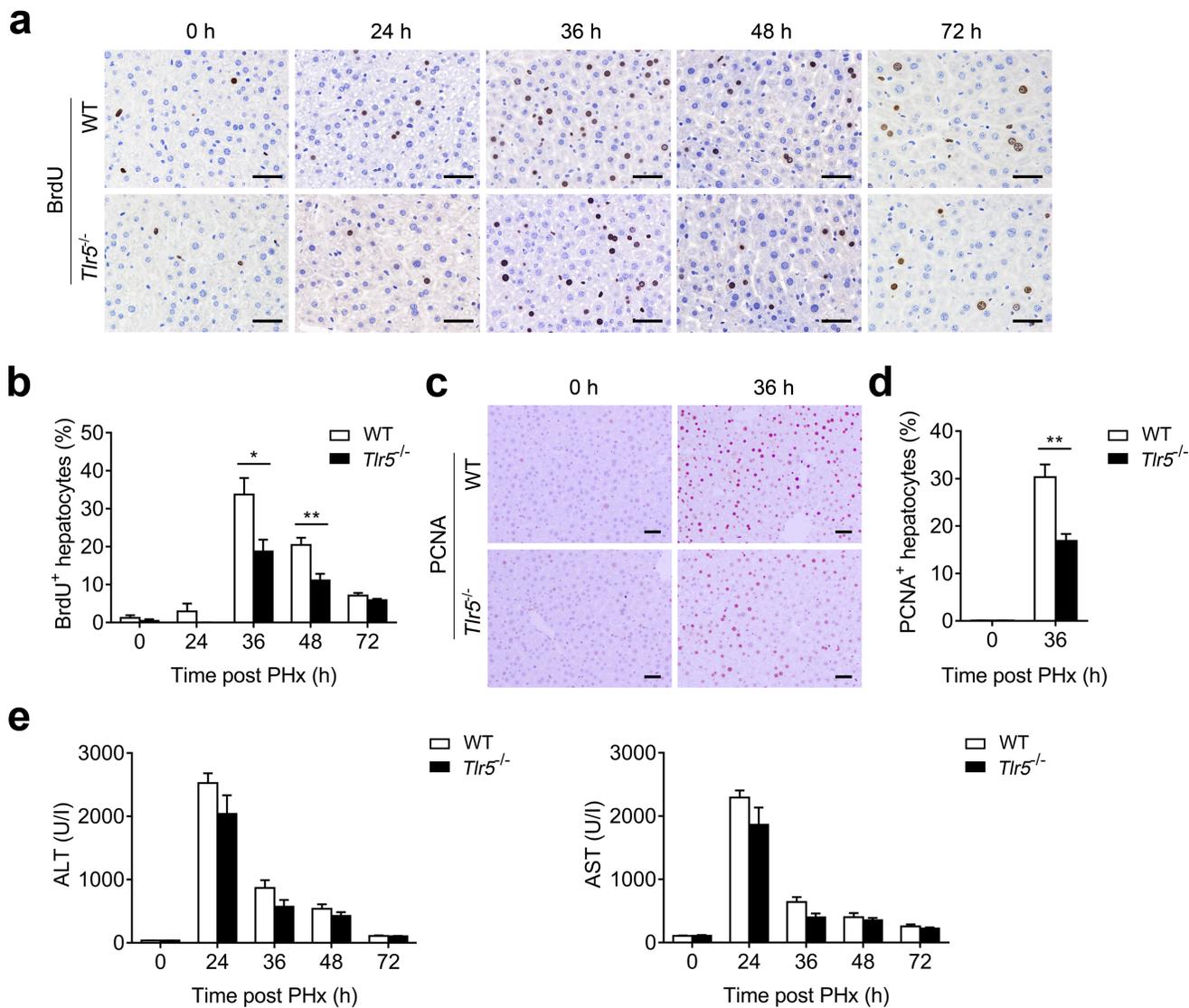


Figure 2

TLR5 deficiency attenuates hepatocyte proliferation following PHx. Proliferation was measured by BrdU and PCNA immunohistochemistry staining. Representative IHC staining images were shown (a and c), and the percentage of BrdU-positive cells (b) and PCNA-positive cells (d) were counted. e. Serum ALT and

AST levels of WT and *Tlr5*^{-/-} mice subjected to PHx. Results were expressed as means ± SEM. Data are derived from two or three independent experiments with similar results. n = 5-6.

Figure 3

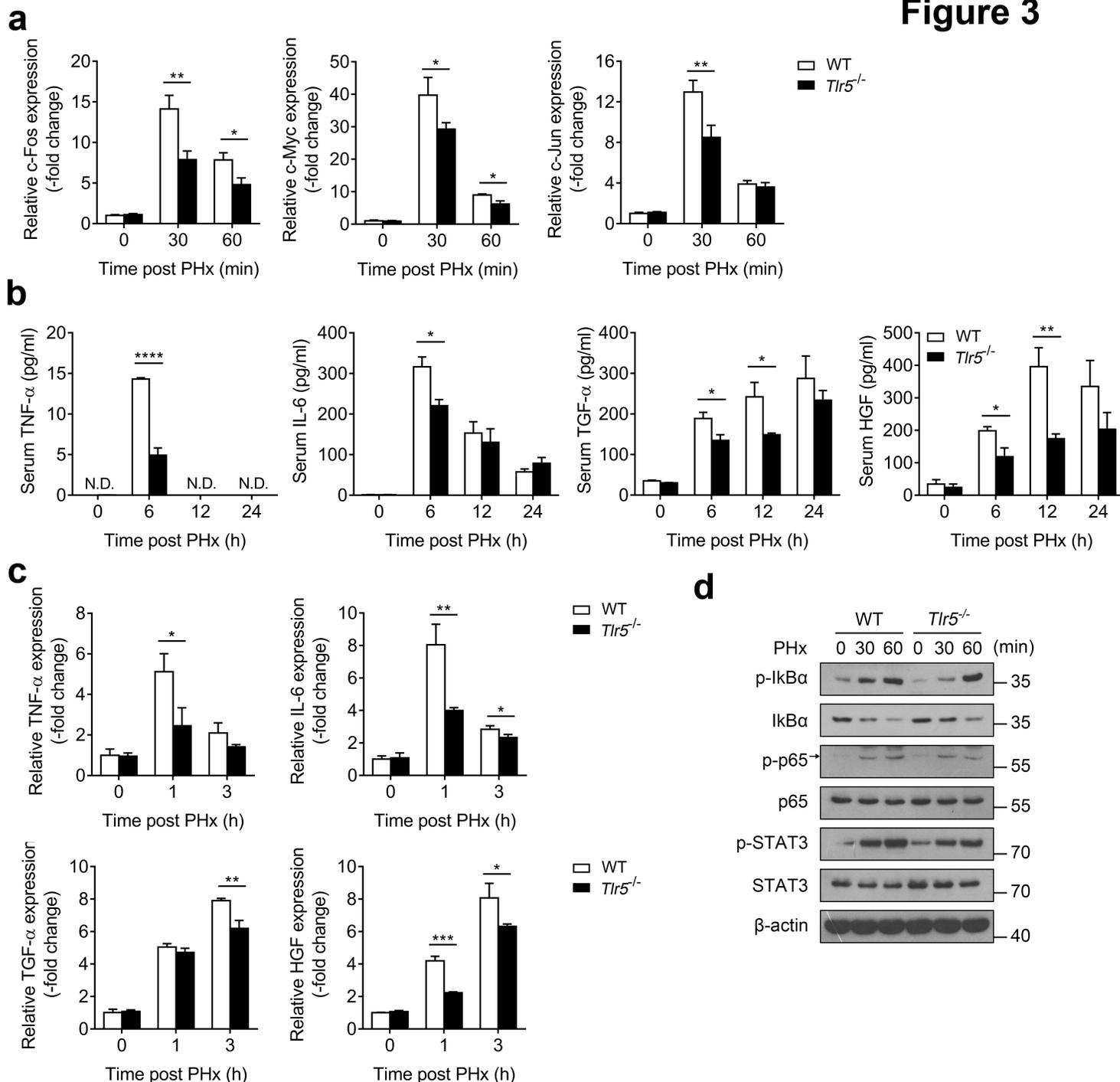


Figure 3

TLR5 deficiency suppresses hepatocyte priming in PHx-induced liver regeneration. a. Relative mRNA levels of hepatic c-fos, c-myc, and c-jun in WT and *Tlr5*^{-/-} mice at the indicated times after PHx. b. Serum TNF-α, IL-6, TGF-α, HGF were measured in regenerating WT and *Tlr5*^{-/-} mice liver. c. Quantification of hepatic TNF-α, IL-6, TGF-α, and HGF mRNA expression in WT and *Tlr5*^{-/-} mice at the

indicated times after PHx. d. Western blot analysis of the indicated target proteins in WT and *Tlr5*^{-/-} mice liver after PHx with β -actin as the loading control. Panel a and c show means \pm SD. Results are representative of three independent experiments. Panel b shows means \pm SEM. Data are derived from two or three independent experiments with similar results. n = 4-6. Western blot data in panel d is representative of three independent experiments. N.D., not detected.

Figure 4

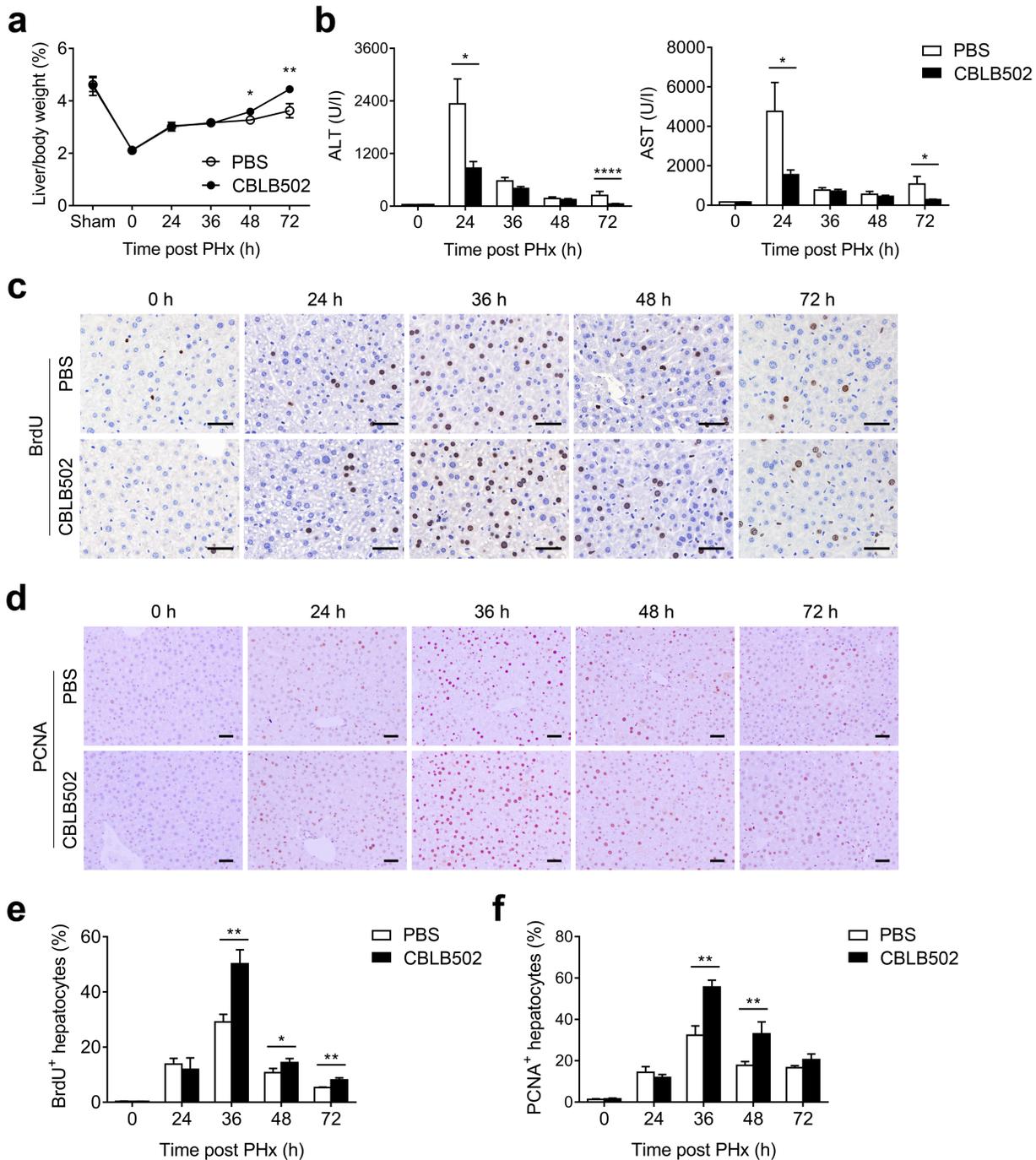


Figure 4

The TLR5 agonist CBLB502 enhances hepatocyte proliferation in mice following PHx. Liver to body weight ratio (a), serum ALT and AST levels (b) of PBS and CBLB502 treated mice after PHx. Hepatocyte proliferation was measured by BrdU (c) and PCNA (d) immunohistochemistry staining. Percentage of BrdU-positive cells (e) and PCNA-positive cells (f) were counted at the indicated times after PHx. Results were expressed as means \pm SEM. Data are derived from two or three independent experiments with similar results. n = 6-12.

Figure 5

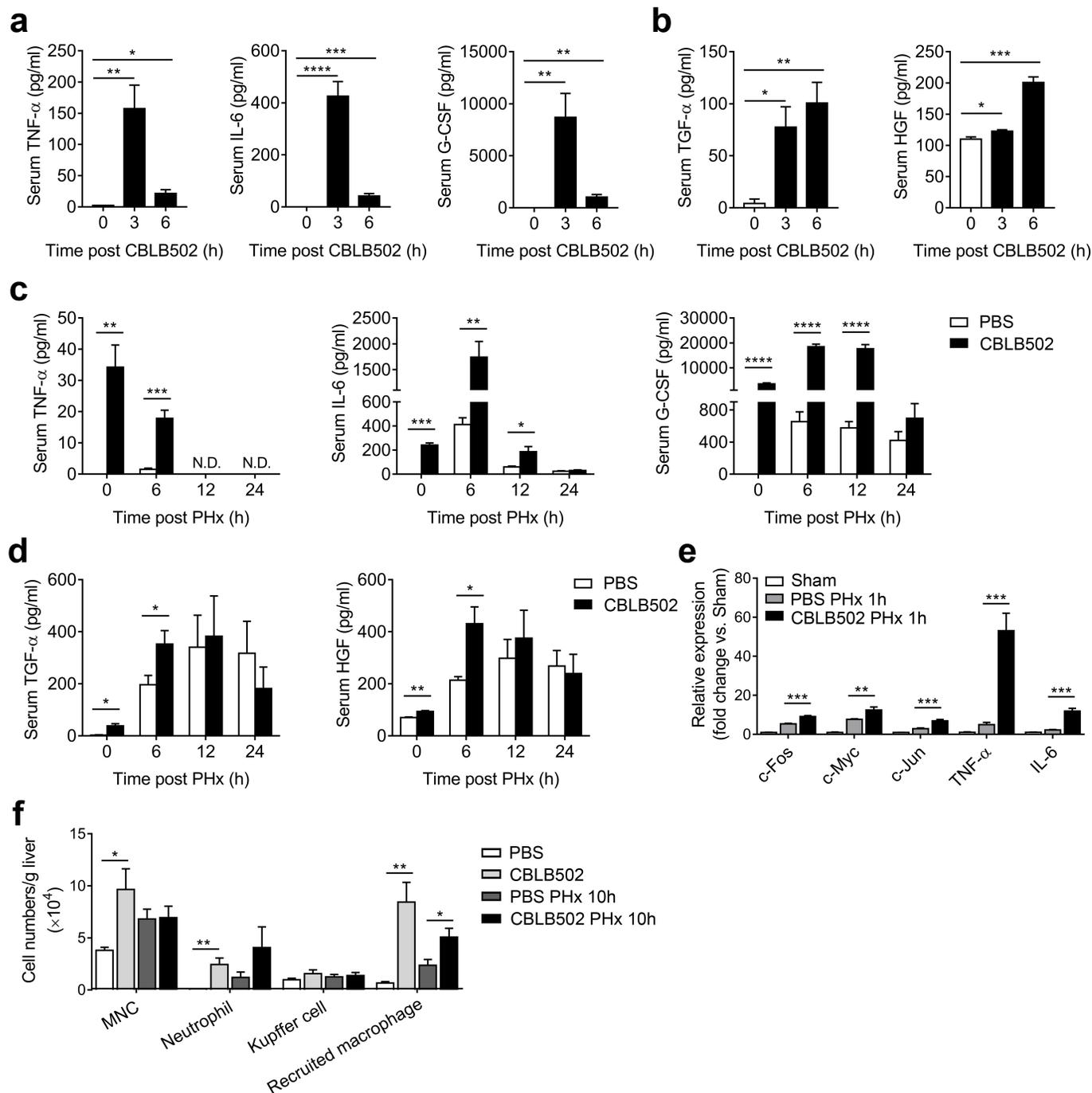
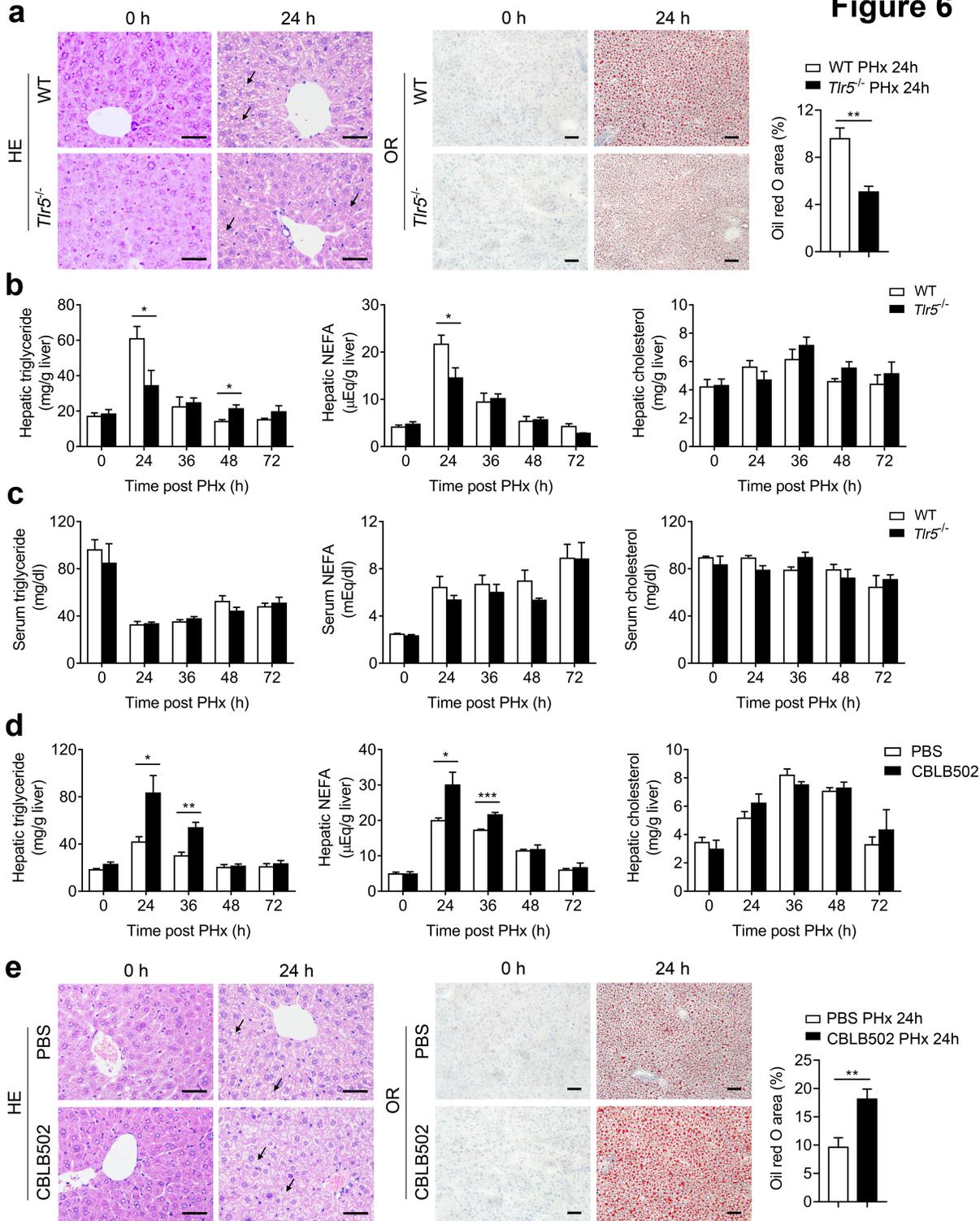


Figure 5

Enhanced inflammatory response in CBLB502-pretreated mice after PHx. Serum TNF- α , IL-6, G-CSF (a), TGF- α , HGF (b) in CBLB502 treated mice at the indicated times. Serum TNF- α , IL-6, G-CSF (c), TGF- α , and HGF (d) were measured in regenerating PBS and CBLB502 treated mice liver after PHx. e. Relative mRNA levels of hepatic c-Fos, c-Myc, c-Jun, TNF- α , and IL-6 in PBS and CBLB502 treated mice upon PHx. f. Cell counts of liver MNCs, neutrophils, KCs, and recruited macrophages in PBS and CBLB502 treated mice following sham surgery or PHx. Panel e shows means \pm SD. Data are representative of three independent experiments. Panel a-d, f show means \pm SEM. Data are derived from two or three independent experiments with similar results. n = 3-7. N.D., not detected.

Figure 6**Figure 6**

TLR5 signaling contributes to hepatic lipid accumulation induced by PHx. a. H&E staining, Oil Red O, quantification of positive areas of WT and *Tlr5*^{-/-} mice livers upon PHx. Triglyceride, NEFA, and cholesterol levels in liver extracts (b) and serum (c) were measured in WT and *Tlr5*^{-/-} mice at the indicated times after PHx. d. Analysis of hepatic triglyceride, NEFA, and cholesterol level in PBS and CBLB502 treated mice upon PHx. e. H&E staining, Oil Red O, quantification of positive areas of PBS and

CBLB502 treated mice livers following PHx. Black arrows indicate hepatic lipid. Results were expressed as means \pm SEM. Data are derived from two or three independent experiments with similar results. n = 5-7.

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