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TLR3 promotes hepatocyte proliferation after partial hepatectomy by stimulating uPA expression and the release of tissue-bound HGF

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Abstract

TLR3 is implicated in anti-viral immune responses, but may also act as a sensor of tissue damage in the absence of infection. Here, we provide evidence for an essential role of TLR3 in liver regeneration after an acute loss of tissue due to partial hepatectomy. Mice lacking TLR3 had a severe and sustained defect in the restoration of liver tissue with reduced liver-to-body weight ratios even after an extended recovery period of 2 weeks. Hepatocyte cell cycle progression into S phase was impaired in TLR3-deficient mice. Mechanistic analyses revealed that TLR3-deficient mice had markedly reduced systemic levels of active HGF, but had increased amounts of inactive tissue-bound HGF. Importantly, expression of uPA, which orchestrates the processing and release of HGF from the hepatic extracellular matrix, was reduced in regenerating livers of TLR3-deficient mice. In addition, expression of the HGF maturation factor HGFAC was transiently diminished in TLR3-deficient mice. In vitro, engagement of TLR3 directly stimulated expression of uPA by hepatic stellate cells. Thus, TLR3 supports liver regeneration through upregulation of uPA, which promotes the release of preformed HGF from extracellular matrix stores.

KEYWORDS

HGF, liver regeneration, stellate cells, TLR3, urokinase-type plasminogen activator

1 | INTRODUCTION

The liver has a high regenerative capacity and may rapidly restore its original organ mass and function after an acute loss of tissue.[1-3] Liver regeneration after partial hepatectomy requires the complex interplay of the innate system and growth factor networks. The initial phase of liver regeneration after partial hepatectomy is known to involve the activation of Toll-like receptors. Thus, production of the cytokines IL-6 and TNFα is abrogated in mice lacking the TLR signaling adapter MyD88.[4-6] Both IL-6 and TNFα are not directly mitogenic for hepatocytes, but may prime hepatocytes for cell

Abbreviations: HGF, hepatocyte growth factor; HGFAC, hepatocyte growth factor activator; PAI-1, plasminogen activator inhibitor type-1; αSMA, α-smooth muscle actin; TGF-β1, transforming growth factor-β1; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

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cycle entry and promote hepatocyte survival. Genetic ablation of individual MyD88-coupled TLRs yielded only minor effects suggesting that signals mediated by several TLRs may converge to activate the MyD88 pathway. The receptors for HGF and the EGF family are considered crucial for driving subsequent cell cycle progression of hepatocytes. Genetic inactivation of either the HGF receptor c-Met or the EGF receptor impaired the entry of hepatocytes into the S-phase of the cell cycle and delayed the recovery of liver weight following partial hepatectomy.[7-10] When c-Met and the EGF receptor were both inactivated, liver regeneration after partial hepatectomy was completely blocked indicating that the mitogenic pathways activated through these receptors have overlapping functions.[11]

TLR3 exhibits a broad expression pattern in the liver including hepatocytes, stellate cells, Kupffer cells, and sinusoidal endothelial cells.[12-15] TLR3 is located in endolysosomes and recognizes dsRNA released from diverse sources including microbial pathogens, commensal bacteria and damaged host cells.[16-19] Binding of dsRNA causes TLR3 dimerization and recruitment of the adapter protein TRIF, but not MyD88, to the cytosolic domain of TLR3. TRIF associates with TRAF6 and RIP1 leading to the activation of NF-κB. Alternatively, TRIF may bind TRAF3 to activate IRF3 and initiate a type-I IFN response. Efficient signaling through TLR3 requires cleavage of its extracellular domain by acid-dependent proteases, thereby limiting TLR3 activation to endolysosomes.[20] In addition, TLR3-stimulated interaction of TRIF with RIP1 or RIP3 may activate caspase-8 leading to the induction of apoptosis or programmed necrosis.[21-25]

In the present study, we show that TLR3 is essential to fully restore liver tissue after partial hepatectomy. Defective liver regeneration in TLR3-deficient mice was linked with an impaired hepatocyte cell cycle progression into the S-phase. The lack of TLR3 caused a strong reduction of systemic HGF levels during the proliferation phase of hepatocytes, while tissue-bound HGF in liver was elevated. Importantly, TLR3 was found to be required for normal hepatic expression of uPA during liver regeneration in vivo and stimulated uPA expression in hepatic stellate cells in vitro. Thus, TLR3 appears to promote liver regeneration after partial hepatectomy through the induction of uPA and the release of HGF from the hepatic extracellular matrix.

2 | MATERIALS AND METHODS

2.1 | Animals

TLR3^{OFF} mice were generated by insertion of a floxed transcriptional termination element between exons 3 and 4 of the *Tlr3* gene, thereby generating a whole body knockout (Figure S1A). TLR3^{OFF} mice were backcrossed to the C57BL/6N background for at least 10 generations and maintained in a specified pathogen-free facility (Charles River, Calco, Italy). C57BL/6N wild-type control mice were obtained from Charles River. All animal experiments were institutionally approved by the government of Upper Bavaria (license 55.2-2532.Vet_02-15-147) and were performed in accordance with the guidelines and regulations.

2.2 | Partial hepatectomy

Male mice at the age of 10-12 weeks were subjected to two-thirds partial hepatectomy according to standard procedures.[26] According to this protocol, ligation and resection of the median lobe and the left lateral lobe was performed separately. Mice were anesthetized with isoflurane. Partial hepatectomies were performed between 8 and 10 AM. After the indicated time periods, mice were sacrificed and remnant livers were collected for further analyses.

2.3 | Liver histology and immunohistochemistry

For detection of hepatocyte proliferation, mice were given ip injections of 100 μg/g BrdU (Roche Diagnostics, Penzberg, Germany) 2 hours before sacrifice. Liver samples were fixed in 4% of paraformaldehyde followed by dehydration and embedded in paraffin. Sections were incubated with anti-BrdU monoclonal antibody (Merck Millipore, Billerica, MA) and stained using the Dako EnVision^{+} System (Agilent Technologies, Santa Clara, CA). Stained sections were analyzed using an Axiolab attached to an AxioCamMRc5 5MPx camera, an CP-Achromat 5x/0.12 NA, an EC-Plan-Neofluar 10x/0.3 NA and an Achroplan 20x/0.45 NA objective (all Zeiss Microscopy, Jena, Germany). For quantification, five random high-power fields for each animal were counted and the fraction of stained hepatocyte nuclei was calculated using the AxioVision Software (Zeiss Microscopy).

2.4 | Western blotting

Liver samples were homogenated in a lysis buffer containing 1% of Triton X-100, 150 mM NaCl, 20 mM Tris-HCl pH 7.5 and protease and phosphatase inhibitors using a TissueLyser II instrument (Qiagen, Hilden, Germany). Lysates (40 μg protein) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were incubated with primary antibodies directed against phosphorylated Rb (S807/S811), phosphorylated

STAT3 (Y705), IκBα, GAPDH (all from Cell Signaling Technology, Danvers, MA), HGF (BioRad Laboratories, Hercules, CA), uPA (R&D Systems, Minneapolis, MI), CDK1 or β-tubulin (both from Abcam, Cambridge, UK). Second stage goat-anti-rabbit-HRP was obtained from Jackson ImmunoResearch. Antibody binding was visualized using the Pierce ECL western blotting detection system (ThermoFisher Scientific, Waltham, MA) and a ChemStudio Plus instrument (Analytik Jena, Jena, Germany). Densitometric analyses were performed using the ImageJ software (http://rsb.info.nih.gov/ij).

2.5 | Quantitative reverse transcriptase PCR

Liver samples were snap frozen in liquid nitrogen immediately after explanation and stored in −80°C until use. RNA was prepared using the RNeasy mini kit (Qiagen). First-strand cDNA was synthesized from 2 μg total RNA using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative RT-PCR analyses were performed using the Universal Probe Library (Roche Diagnostics). The primers were: mouse cyclin A2 sense, 5'-CTT GGC TGC ACC AAC AGT AA-3'; mouse cyclin A2 antisense, 5'-CAA ACT CAG TTC TCC CAA AAA CA-3'; mouse cyclin B1 sense, 5'-GCT TAG CGC TGA AAA TTC TTG-3'; mouse cyclin B1 antisense, 5'-TCT TAG CCA GGT GCT GCA TA-3'; mouse cyclin D1 sense, 5'-TTT CTT TCC AGA GTC ATC AAG TGT-3'; mouse cyclin D1 antisense, 5'-TGA CTC CAG AAG GGC TTC AA-3'; mouse cyclin E1 sense, 5'-TTT CTG CAG CGT CAT CCT C-3'; mouse cyclin E1 antisense, 5'-TGG AGC TTA TAG ACT TCG CAC A-3'; mouse uPA sense, 5'-GCC TTG GTG AAA AAC TC-3'; mouse uPA antisense, 5'-CAC GCA TAC ACC TCC GTT C-3'; human uPA sense, 5'-TTG CTC ACC ACA ACG ACA TT-3'; human uPA antisense, 5'-GGC AGG CAG ATG GTC TGT AT-3'; mouse tPA sense, 5'-TCA GTG CCT GTC CGA AGT T-3'; mouse tPA antisense, 5'-GCA CTG GCA GAC AAA GTC AG-3'; mouse PAI-1 sense, 5'-AGG ATC GAG GTA AAC GAG AGC-3'; mouse PAI-1 antisense, 5'-GCG GGC TGA GAT GAC AAA-3'; mouse HGFAC sense, 5'-AAC ACA CAA CTA TGA CCG AGA CC-3'; mouse HGFAC antisense, 5'-GCA CAA GGG TCA AGG ATA GC-3'; human αSMA sense, 5'-CCT ATC CCC GGG ACT AAG AC-3'; human αSMA antisense, 5'-AGG CAG TGC TGT CCT CTT CT-3'; human TGF-β1 sense, 5'-ACT ACT ACG CCA AGG TCA C-3'; human TGF-β1 antisense, 5'-TGC TTG AAC TTG TCA TAG ATT TCG-3'; mouse TLR3 sense, 5'-GAT ACA GGG ATT GCA CCC ATA-3'; mouse TLR3 antisense, 5'-TCC CCC AAA GGA GTA CAT TAG A-3'. RNA levels were normalized to those of β-actin and are depicted as relative quantification ($2^{-\Delta ct}$) or as fold difference relative to liver samples of untreated mice. Accumulation of PCR amplicons was quantified on a LightCycler 480 Real-Time PCR system (Roche Diagnostics).

2.6 | IL-6 and HGF serum levels

IL-6 and HGF protein concentrations in serum were determined using Quantikine ELISA kits (R&D Systems). Assays were performed according to manufacturer's instructions.

2.7 | Statistical analysis

All data are presented as mean ± standard error. Statistical differences were analyzed using the two-tailed Student's *t* test or the Mann-Whitney test. A difference between experimental groups was considered significant, when the *P* value was less than 0.05.

3 | RESULTS

3.1 | Lack of TLR3 prevents complete recovery of liver tissue after partial hepatectomy

The present study examines the role of TLR3 for liver regeneration using a model of 70% of partial hepatectomy.[26] We used a novel mutant mouse strain exhibiting global TLR3 deficiency ($TLR3^{OFF}$) that was generated by insertion of a floxed transcriptional termination element between exons 3 and 4 of the *Tlr3* gene (Figure S1A). Genetic inactivation of TLR3 was confirmed by the lack of TLR3 protein expression of bone marrow-derived macrophages generated from $TLR3^{OFF}$ mice (Figure S1B). IL-6 production of $TLR3^{OFF}$ bone marrow-derived macrophages was abrogated upon stimulation with the TLR3 agonist poly(I:C), but was normal upon stimulation with the TLR9 agonist CpG-DNA (Figure S1C). Importantly, TLR3 mRNA was not detectable in liver samples of $TLR3^{OFF}$ mice (Figure S1D).

Global deletion of TLR3 did not affect liver homeostasis, as histomorphological analyses of livers including hepatocytes, biliary ducts, blood vessels, and immune cell infiltration, did not reveal any differences between untreated wild-type and $TLR3^{OFF}$ mice (Figure S2). Moreover, liver-to-body weight ratios of $TLR3^{OFF}$ mice were similar to those of wild-type controls in the untreated state (Figure 1A). After partial hepatectomy, however, the recovery of liver tissue was substantially impaired in the absence of TLR3. Whereas liver-to-body weight ratios were comparable between wild-type and $TLR3^{OFF}$ mice until 48 hours after partial hepatectomy, $TLR3^{OFF}$ mice had a severely reduced recovery of liver tissue at all later time points and their relative liver weight did

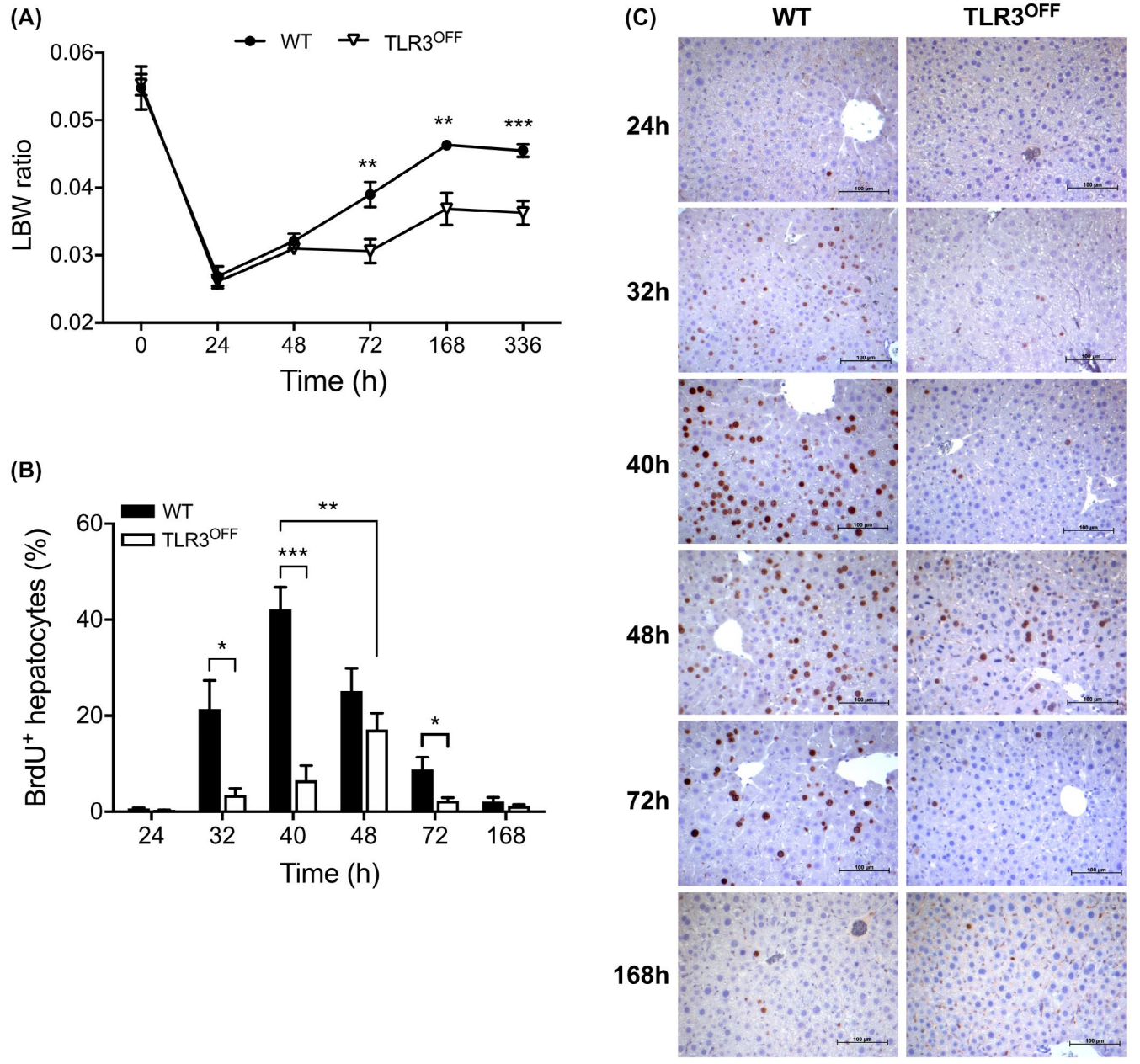

FIGURE 1 TLR3 deficiency severely impairs restoration of liver tissue and hepatocyte proliferation after partial hepatectomy. A, Liver-to-body weight (LBW) ratios were determined at the indicated time points following partial hepatectomy (n = 6 − 9 mice per time point in each group). B, Quantification of the fraction of BrdU-positive hepatocytes (n = 5 − 6 mice per time point in each group) and (C) representative immunohistochemical images are shown. Scale bars represent 100 μm. A, B, Data were pooled from at least 3 independent experiments and were analyzed using the two-tailed Student's *t* test or the Mann-Whitney *U* test. Data are presented as mean ± SEM. **P* < .05, ***P* < .01, ****P* < .001

not fully recover (Figure 1A). Even after 168 and 336 hours, when livers of wild-type mice had fully recovered, the final liver-to-body weight ratio of TLR3^{OFF} mice only reached about 80% of the wild-type level. However, there we did not observe any mortalities in either wild-type or TLR3^{OFF} mice (168 and 336 hours experimental groups; n = 7 per group and time point). These results, therefore, indicate that TLR3^{OFF} mice show a delayed liver regeneration after partial hepatectomy and have a persistently reduced liver size even after 14 days.

3.2 | Hepatocyte proliferation is impaired in the absence of TLR3

To uncover potential mechanisms, by which TLR3 promotes liver regeneration, hepatocyte proliferation was investigated using in vivo BrdU incorporation. In wild-type mice, the fraction of proliferating hepatocytes markedly increased from 24 to 40 hours after partial hepatectomy and decreased thereafter (Figure 1B,C). In TLR3^{OFF} mice, the fraction of BrdU-positive hepatocytes was significantly diminished at the 32

and 40 hours time points and the peak level of BrdU incorporation was reached only after 48 hours as compared with 40 hours in wild-type mice. However, even at the 48 hours time point, hepatocyte proliferation in TLR3^{OFF} mice did not reach maximal levels of wild-type hepatocyte proliferation (40 hours time point). Moreover, TLR3^{OFF} livers showed a significantly reduced fraction of BrdU-positive hepatocytes at the 72 hours time point suggesting a premature decline of cell proliferation. These results, therefore, indicate that the lack of TLR3 markedly attenuates hepatocyte proliferation and, in addition, shortens their proliferation phase.

To identify the mechanisms, by which TLR3 promotes hepatocyte proliferation the expression of various cell cycle regulators was determined. As shown in Figure 2A,B, mRNA levels of cyclins D1 and E1, which are upregulated during the late G_1-phase of the cell cycle, were reduced in TLR3^{OFF} as compared with wild-type mice from 24 until 40 hours after partial hepatectomy. Notably, the early peak of cyclin D1 and E1 expression that was observed in wild-type mice at the 32 hours time point was not present in TLR3^{OFF} mice indicating that in the absence of TLR3 the induction of early cell cycle regulators is blunted. Cell cycle progression beyond the G_1/S restriction point requires inactivation of Rb protein through hyperphosphorylation.[27] Our results show that phosphorylation of Rb protein was strongly diminished in livers of TLR3^{OFF} mice from 32 until 48 hours after partial hepatectomy (Figure 2C). Of note, maximal levels of both cyclin E1 expression and phosphorylation of Rb protein were significantly lower than those observed in wild-type livers (Figure 2B,C). The results in Figure 2D further demonstrate that upregulation of the kinase CDK1, which becomes activated by A- and B-type cyclins and acts as an essential driver of the mammalian cell cycle, was also impaired in TLR3-deficient mice at 32 and 40 hours after partial hepatectomy. Expression of cyclin A2 was markedly diminished in TLR3^{OFF} as compared with wild-type mice at the 32, 40, and 72 hours time points (Figure 2E), and expression of cyclin B1 was reduced at 40 hours (Figure 2F). Together, these results indicate that TLR3 is essential for a normal hepatocyte cell cycle progression beyond the G_1/S restriction point in the regenerating liver.

To provide direct evidence for TLR3 activation during liver regeneration, downstream signaling of TLR3 was examined. Ligand binding of TLR3 initiates multiple signaling pathways including activation of NF-κB.[16-19] Livers of wild-type mice showed a significant reduction of IκBα protein levels at 24 and 32 hours after partial hepatectomy, indicating NF-κB activation at these time points (Figure S3). Livers of TLR3^{OFF} mice, however, did not exhibit degradation of IκBα at any time point investigated and had significantly increased IκBα levels as compared with wild-type livers at 24 and 32 hours after partial hepatectomy and, in addition, at the 40 and 72 hours time points. These results, therefore, demonstrate the signaling activity of TLR3 during liver regeneration and reveal an important role of TLR3 for NF-κB activation.

3.3 | IL-6 release and STAT3 activation in regenerating livers are independent of TLR3

The release of IL-6 during the first hours after partial hepatectomy is thought to modulate liver regeneration[1,3,28,29] and is mediated by TLR signaling.[4-6,30] The results in Figure S4A demonstrate that serum IL-6 levels were similar between wild-type and TLR3^{OFF} mice between 2 and 12 hours after partial hepatectomy. Consistent with these findings, wild-type and TLR3^{OFF} livers also showed comparable levels of phosphorylated STAT3, which acts as a signal transducer of the IL-6 receptor (Figure S4B). Thus, IL-6 production and STAT3 activation during the initiation phase of liver regeneration were found to be independent of TLR3.

3.4 | TLR3 deficiency leads to an impaired release of HGF from liver tissue during the proliferation phase of liver regeneration

HGF provides an essential stimulus for hepatocyte proliferation and liver regeneration after toxic liver injury or partial hepatectomy.[7,31,32] As shown in Figure 3A, wild-type and TLR3^{OFF} mice showed a rapid and comparable increase of serum HGF levels during the early phase of liver regeneration (4 and 8 hours time points). However, while serum HGF levels continued to increase between 24 and 40 hours after partial hepatectomy in wild-type controls, they decreased in TLR3^{OFF} mice reaching baseline levels after 40 hours. As a consequence, serum HGF levels were significantly reduced in TLR3^{OFF} as compared with wild-type mice between 24 and 40 hours after partial hepatectomy (Figure 3A). At the 48 and 72 hours time points, HGF serum levels were again comparable between wild-type and TLR3^{OFF} mice. Notably, the time period of reduced serum HGF levels in TLR3^{OFF} mice directly coincided with impaired hepatocyte proliferation.

The HGF protein is deposited in the extracellular matrix of the liver and is proteolytically released during liver regeneration.[33,34] Therefore, we also determined HGF levels in tissue extracts of TLR3^{OFF} and wild-type livers. The results depicted in Figure 3B demonstrate that, in wild-type mice, HGF α-chain levels in liver tissue rapidly increased at 4 and 8 hours after partial hepatectomy, followed by a decrease below the levels of untreated mice at 24 and 32 hours. Thereafter, hepatic levels of the HGF α-chain returned to baseline levels in wild-type mice. In TLR3^{OFF} mice, we also observed an increase of hepatic HGF α-chain levels at the 4 and 8 hours time points, which was comparable to that of wild-type controls. However, TLR3^{OFF} mice showed only a

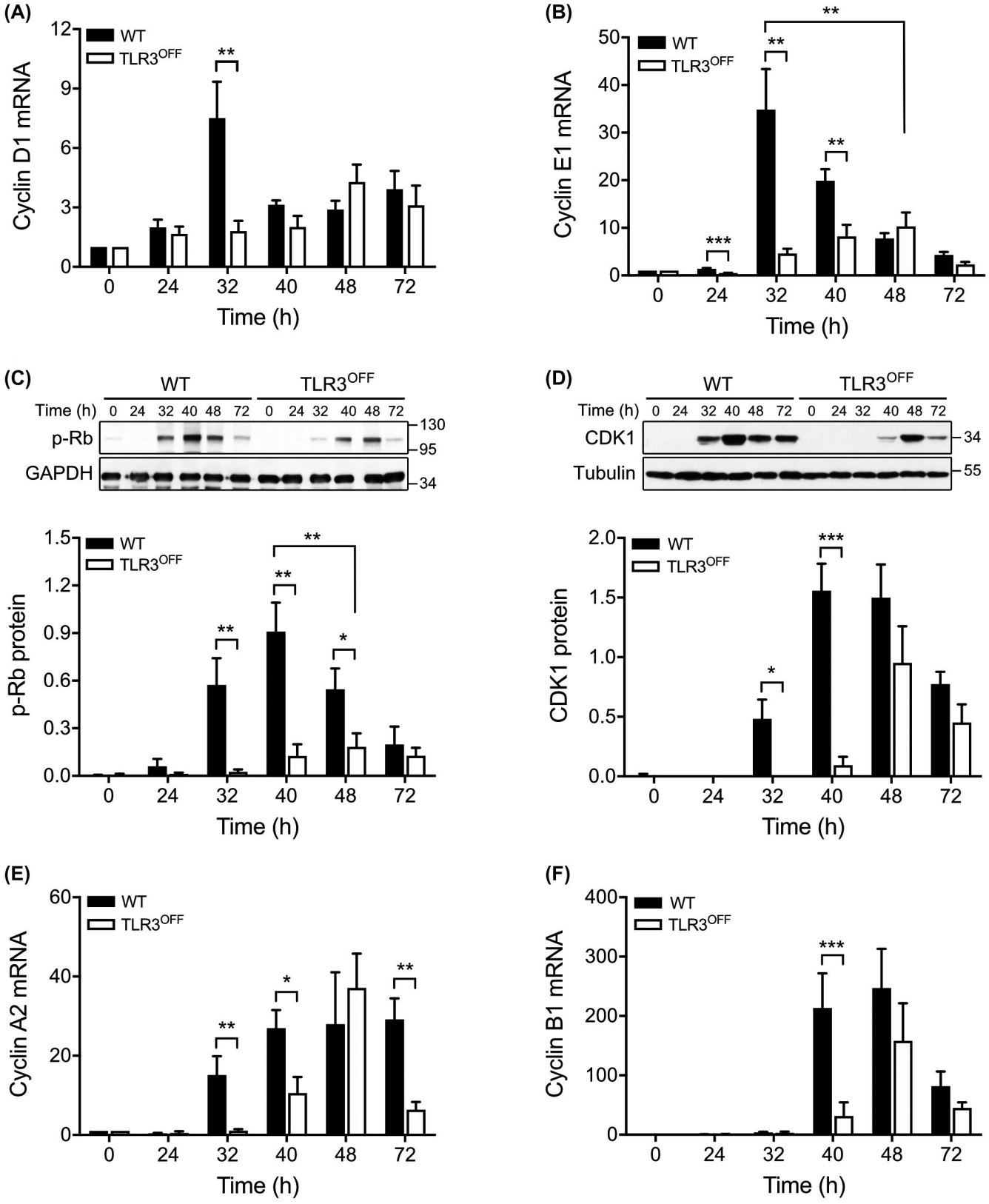

FIGURE 2 Hepatocyte cell cycle progression is impaired in TLR3^{OFF} mice. A, B, Hepatic mRNA levels of cyclins D1 and E1 were normalized to those of β-actin and are presented as fold difference relative to untreated controls. C, D, Total protein extracts of liver samples were analyzed by Western blotting using antibodies against phosphorylated Rb protein, CDK1, and GAPDH or β-tubulin as loading controls. Representative gels and densitometric analyses are shown. E, F, Hepatic mRNA levels of cyclins A2 and B1 were normalized to those of β-actin and are given as fold difference relative to untreated controls. For each time point and group samples from 5-6 independent mice were analyzed. Data were pooled from at least 3 independent experiments and were analyzed using the two-tailed Student's *t* test or the Mann-Whitney *U* test. Data are presented as mean ± SEM $*P < .05$, $**P < .01$, $***P < .001$

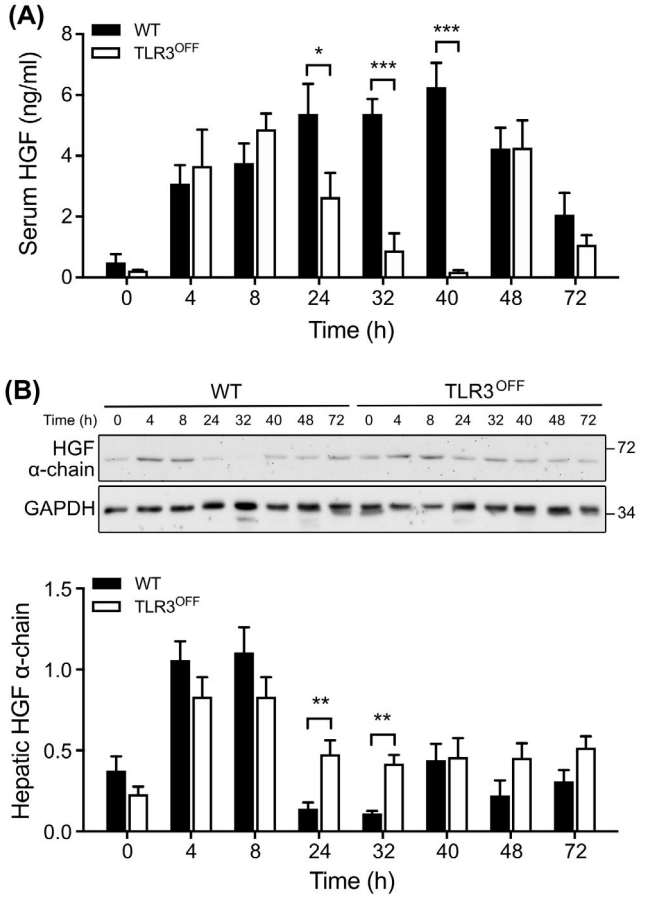

FIGURE 3 HGF levels are reduced in serum and increased in hepatic tissue in TLR3 deficient mice. A, Serum levels of HGF were determined at the indicated time points after partial hepatectomy by ELISA. B, Tissue-bound levels of the HGF α-chain were determined by Western blotting using GAPDH as a loading control. For each time point and group samples from 6-8 independent mice were analyzed. Data were pooled from at least 3 independent experiments and were analyzed using the two-tailed Student's *t* test or the Mann-Whitney *U* test. Data are presented as mean ± SEM. $*P < .05$, $**P < .01$, $***P < .001$

weak reduction of the tissue-bound HGF α-chain at 24 and 32 hours after partial hepatectomy resulting in significantly increased hepatic HGF α-chain levels as compared with wild-type controls at 24 and 32 hours time points (Figure 3B). At later time points (40-72 hours), hepatic HGF α-chain levels were comparable between wild-type and TLR3^{OFF} mice. These findings, therefore, suggest that the release of HGF from liver tissue is impaired in TLR3^{OFF} mice during the early proliferation phase of hepatocytes.

3.5 | TLR3 promotes expression of uPA in the regenerating liver in vivo and stimulates hepatic stellate cell uPA production in vitro

In the regenerating liver, uPA is crucial for orchestrating the proteolytic release and thereby activation of HGF.[35-37] Importantly, reduced serum HGF levels in TLR3^{OFF} mice were associated with an impaired expression of uPA from 24 to 40 hours after partial hepatectomy, while mRNA levels of tPA and the inhibitor PAI-1 were comparable between wild-type and TLR3^{OFF} mice (Figure 4A). Expression of the HGF-activating serine protease HGFAC was also significantly reduced in TLR3^{OFF} mice 32 hours after partial hepatectomy (Figure 4A). In accordance with the mRNA expression of uPA, protein levels of uPA were also significantly diminished in livers of TLR3^{OFF} mice as compared with wild-type controls between 24 and 40 hours after partial hepatectomy (Figure 4B). These results, therefore, reveal a novel function of TLR3 for promoting the expression of uPA in regenerating livers. Moreover, the findings suggest that TLR3 enhances the release of HGF from the hepatic extracellular matrix through upregulation of uPA and, in part, HGFAC.

Hepatic stellate cells express functional TLR3[13,14] and synthesize various components of the plasminogen-activating system.[38] To find out whether hepatic stellate cells may be involved in controlling HGF release by uPA downstream of TLR3, we directly stimulated the hepatic stellate cell line LX-2 with the TLR3 agonist poly(I:C). Upon TLR3 activation of LX-2, but not the hepatoma cell line HepG2, significantly increased uPA expression (Figure 5). In contrast to uPA, poly(I:C) treatment of LX-2 cells did not influence their expression of αSMA and TGF-β1 suggesting that TLR3 engagement does not lead to a global stellate cell activation. Instead, TLR3 appears to specifically stimulate uPA expression in hepatic stellate cells.

4 | DISCUSSION

Apart from its role in the host defense against viral infections,[39] TLR3 serves as an endogenous sensor of tissue injury by recognizing dsRNA that is released from damaged host cells.[40-43] Following tissue injury, TLR3 may enhance the inflammatory response and stimulate the production of cytokines like IL-6 that promote tissue repair.[40-43] In the present report, we describe an essential role of TLR3 for liver regeneration after an acute loss of liver tissue due to 70% of hepatectomy. We found that, even when the recovery period after partial hepatectomy was extended for up to 2 weeks, final liver-to-body weight ratios of hepatectomized TLR3^{OFF} mice were significantly lower than those of wild-type controls. Cell cycle analyses indicated that TLR3 is required for a normal hepatocyte cell cycle progression beyond the G_1/S restriction point. However, TLR3 did not affect the production of IL-6 or the activation of STAT3 during the initiation phase of liver regeneration. Instead, our studies identify TLR3 as a novel regulator of uPA expression in the regenerating liver. The data support the concept that, during the proliferation phase of liver regeneration, TLR3 promotes the

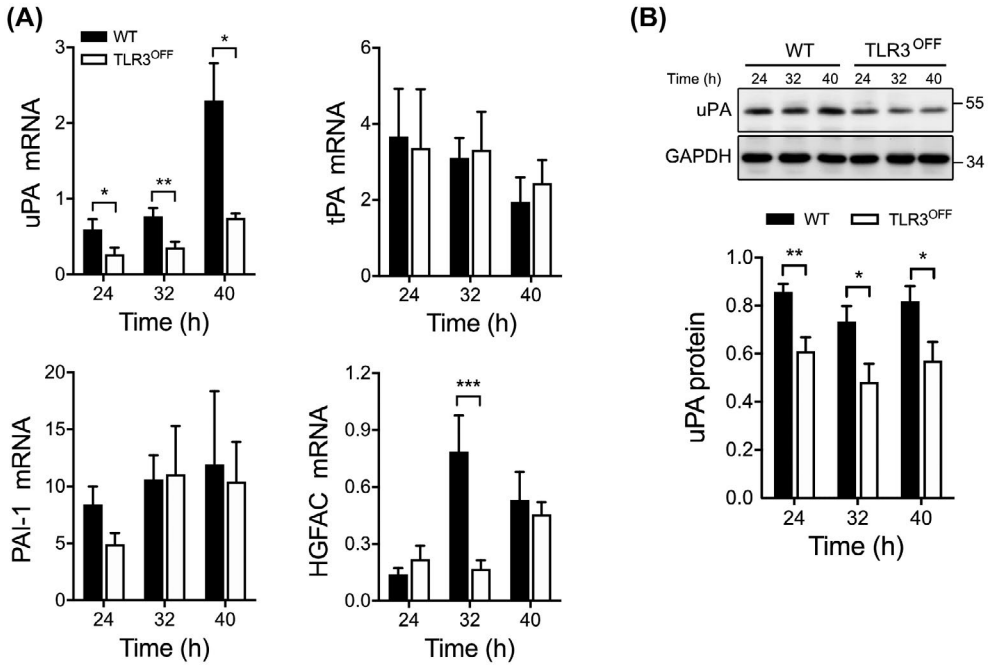

FIGURE 4 TLR3 is required for normal expression of uPA in regenerating livers. A, Hepatic mRNA expression of uPA, tPA, PAI-1, and HGFAC were determined at the indicated time points after partial hepatectomy. mRNA levels were normalized to those of β-actin and are given as fold difference relative to untreated controls. B, Protein levels of uPA in regenerating livers were determined by Western blotting using GAPDH as a loading control. For each time point and group samples from 6-8 independent mice were analyzed. Data were pooled from at least 3 independent experiments and were analyzed using the two-tailed Student's *t* test or the Mann-Whitney *U* test. Data are presented as mean ± SEM. $*P < .05$, $**P < .01$, $***P < .001$

expression of uPA in hepatic stellate cells, which leads to the release of HGF from extracellular matrix stores, and stimulates hepatocyte proliferation.

HGF acts as an important growth factor that stimulates hepatocyte cell cycle progression and promotes liver regeneration and repair.[7,10,31] Genetic inactivation of the HGF receptor c-Met was found to result in a prolonged defect in the recovery of liver tissue after partial hepatectomy and a diminished hepatocyte cell cycle progression into S phase, a phenotype that is similar to that of TLR3^{OFF} mice. In the present study, we also show that hepatectomized TLR3^{OFF} mice have markedly reduced serum HGF levels during a time period that is characterized by extensive hepatocyte proliferation in wild-type mice. Thus, defective liver regeneration and impaired hepatocyte proliferation in TLR3^{OFF} mice are likely to result from low levels of soluble HGF.

Newly synthesized HGF is deposited in the extracellular matrix of the liver,[33] from which it must be proteolytically released and processed to stimulate hepatocyte proliferation after partial hepatectomy.[34] The serine protease uPA is known to orchestrate the release of HGF from the hepatic extracellular matrix and its subsequent processing during liver regeneration.[35,36] Accordingly, mice lacking uPA show an impaired hepatocyte proliferation and HGF maturation after liver injury.[37,44] In the present report, we show that, during the proliferation phase of hepatocytes, TLR3^{OFF} mice showed reduced serum HGF levels, increased levels of tissue-bound HGF and an impaired hepatic expression of uPA. In contrast, TLR3 did not appear to influence the expression of tPA or PAI-1. Notably, engagement of TLR3 directly stimulated expression of uPA in a hepatic stellate cell line in vitro. Together, these findings indicate that TLR3 is required for the persistent production of uPA and the release of HGF from the extracellular matrix of regenerating livers. In this context, hepatic stellate cells may act as cellular sources of TLR3-stimulated uPA expression.

HGF is released in consecutive stages after partial hepatectomy.[3,45,46] Within the first hours matrix-bound HGF is released in an uPA-dependent manner. After about 24 hours, new HGF is synthesized by hepatic stellate cells. In the present study, the effects of TLR3 on systemic HGF levels and uPA expression were observed between 24 and 40 hours after hepatectomy. Thus, these findings are consistent with the view that newly synthesized HGF is also transiently deposited in the extracellular matrix and requires release by uPA. At about 2-3 days after hepatectomy, sinusoidal endothelial cell precursors from the bone marrow accumulate in the regenerating liver and represent a major source of HGF.[45,46] It is, therefore, tempting to speculate that the increase of HGF levels in TLR3^{OFF} mice at 48 hours after hepatectomy is due to a TLR3-independent HGF production by these endothelial precursors.

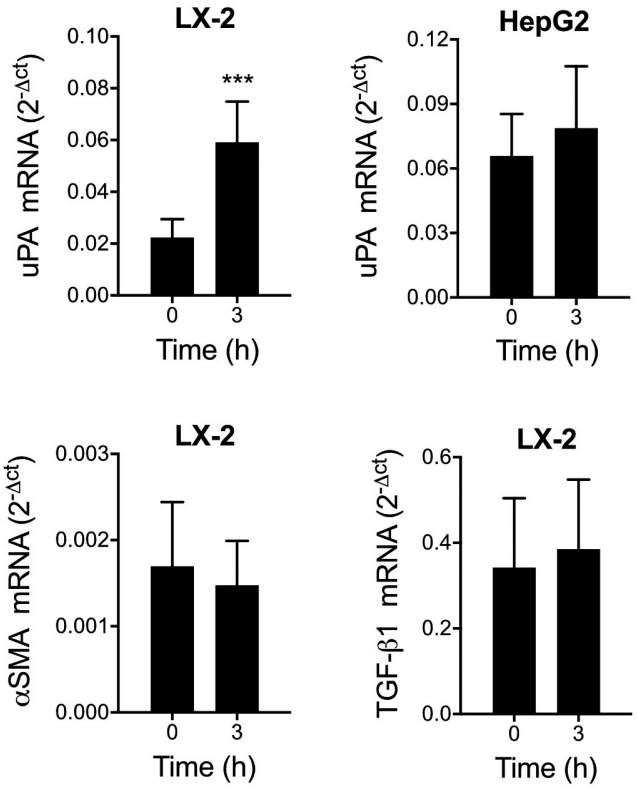

FIGURE 5 TLR3 stimulates uPA expression in hepatic stellate cells. LX-2 hepatic stellate cells and HepG2 hepatoma cells were serum starved overnight and stimulated with 50 μg/mL poly(I:C) for the indicated time periods. Cellular mRNA levels of uPA, αSMA and TGF-β1 are shown relative to those of β-actin. Data were pooled from 5-6 independent experiments and were analyzed using the two-tailed Student's *t* test or the Mann-Whitney *U* test. Data are presented as mean ± SEM. ***$P < .001$

Hepatic stellate cells were shown to express various components of the plasminogen-activating system including uPA and PAI-1.[38] In the present report, we identify TLR3 as a previously unrecognized inducer of hepatic stellate cell uPA expression. Stimulation of stellate cells through TLR3 did not influence the expression of αSMA and TGF-β1 suggesting that engagement of TLR3 does not lead to a global activation of hepatic stellate cells. Previous studies have suggested that hepatic stellate cells may promote liver regeneration through various pathways. Thus, after partial hepatectomy, activation of the β-PDGF receptor in hepatic stellate cells is important to dampen liver injury[47] and IL-6 trans-signaling causes hepatic stellate cells to produce HGF.[48] Our findings, therefore, indicate that activation of uPA expression in response to TLR3 engagement represents a novel pathway, through which hepatic stellate cells support liver regeneration.

Hepatic stellate cells represent approximately 10% of all resident liver cells[49] and express multiple Toll-like receptors including functional TLR3.[13,50] In vitro experiments using primary hepatic stellate cells and the hepatic stellate cell line LX-2 have shown that TLR3 is stimulated by exosomes that were derived from damaged hepatocytes.[14] In the serum of mice subjected to partial hepatectomy, nucleic acid-containing exosomes are highly increased with highest levels being observed after 24 hours.[51] Thus, the release of exosomes into the circulation coincides with the TLR3-dependent phase of uPA expression in the regenerating livers. It, therefore, appears likely that circulating exosomes may serve to stimulate TLR3 in hepatic stellate cells resulting in the production of uPA during the proliferation phase of hepatocytes.

The present report provides evidence that TLR3 is required for triggering normal hepatocyte proliferation after partial hepatectomy. However, when TLR3 is stimulated prior to hepatectomy through exogenous agonists such as injection of poly(I:C) or viral infection, it was found to inhibit liver regeneration.[52,53] It, therefore, appears that the timing of TLR3 activation relative to the loss of liver tissue is crucial for determining outcome. Whereas stimulation of TLR3 through exogenous agonists prior to the insult may inhibit liver regeneration, stimulation of TLR3 through endogenous ligands after initiation of the regenerative response may be essential for an efficient hepatocyte proliferation and the full recovery of liver tissue. Mechanistically, pretreatment of mice with exogenous TLR3 agonists stimulates NK cells to produce IFN-γ leading to the activation of STAT1 and the upregulation of the cell cycle inhibitor p21.[52,53] In contrast, our findings indicate that engagement of TLR3 by endogenous ligands released after partial hepatectomy stimulates liver regeneration through the uPA-HGF pathway.

While our study provides evidence that TLR3 is essential for efficient liver regeneration after partial hepatectomy, a previous report suggests that TLR3 may have an inhibitory activity.[30] It should be considered, however, that our study and that of Zorde-Khvalevsky and coworkers[30] exhibit important technical differences, which may relate to different pathophysiological pathways. Most notably, the study by Zorde-Khvalevsky[30] and coworkers used ketamine/xylazine as anesthetics, whereas isoflurane was used in our study. Anesthesia using ketamine/xylazine has been shown to generate considerably higher hepatotoxicity than isoflurane, which is reported as the least toxic among the commonly used anesthetics.[26] It, therefore, appears likely that toxic effects of ketamine/xylazine may have caused the release of TLR3 ligands from damaged hepatocytes prior to the onset of the regeneration response. Thus, anesthesia with ketamine/xylazine may cause priming of mice through TLR3 similar to preoperative injection of poly(I:C) or viral infection.[52,53] However, under conditions of low hepatotoxicity, stimulation of TLR3 through endogenous ligands released during the course of regeneration may be prevalent. Furthermore, it has been shown that ketamine directly interferes with TLR3 signaling and suppresses the TLR3-mediated activation of microglial cells.[54] It is, therefore, conceivable that ketamine

anesthesia, in addition to priming effects, may also inhibit hepatic stellate cells, and thus, oppose activation of TLR3 during liver regeneration.

In summary, the findings demonstrate that stimulation of TLR3 through endogenous agonists is essential for liver regeneration after partial hepatectomy. TLR3 was identified as a novel inducer of uPA allowing for the mitogenic action of HGF through its release from hepatic extracellular matrix stores during the proliferation phase of hepatocytes. Hepatic stellate cells may represent important targets of TLR3 activity. Thus, engagement of TLR3 appears to be a crucial control element of liver regeneration that may also be relevant in patients with liver disease.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Designed research: B. Holzmann, M. Laschinger, N. Hüser; Performed research: C. Stöß, M. Laschinger, B. Wang, M. Lu, F. Altmayr; Analyzed and interpreted data: C. Stöß, B. Holzmann, M. Laschinger, D. Hartmann, N. Hüser,; Wrote the paper: B. Holzmann, C. Stöß, M. Laschinger, N. Hüser.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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