GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Toll-Like Receptor 4—Independent Carbon Tetrachloride—Induced Fibrosis and Lipopolysaccharide-Induced Acute Liver Injury in Mice

Role of Hepatic Stellate Cells

Sudhir Kumar,^{*†‡} Jiang Wang,[§] Shiva Kumar Shanmukhappa,[¶] and Chandrashekhar R. Gandhi^{*†‡}

From the Departments of Pediatrics* and Pathology and Laboratory Medicine,[¶] Cincinnati Children's Hospital Medical Center, Cincinnati; the Cincinnati VA Medical Center,[†] Cincinnati; and the Departments of Surgery[‡] and Pathology and Laboratory Medicine,[§] University of Cincinnati, Cincinnati, Ohio

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Address correspondence to
Chandrashekhar R. Gandhi,
Ph.D., Department of Pediatrics,
Cincinnati Children's
Hospital Medical Center, 3333
Burnett Ave., Cincinnati,
OH. E-mail: chandrashekhar.gandhi@cchmc.org.

Gram-negative bacterial endotoxin lipopolysaccharide (LPS) is implicated in acute and chronic liver injury; its effects are mediated predominantly via the membrane receptor Toll-like receptor 4 (TLR4). However, TLR4-independent effects of LPS may play important role in hepatic pathophysiology. We investigated carbon tetrachloride (CCl₄)—induced fibrosis and LPS-induced acute liver injury in wild-type (WT) and B6.B10ScN-Tlr4^{lps-del}/JthJ [TLR4-knockout (KO)] mice. Effects of LPS on fibrogenic hepatic stellate cells (HSCs) from WT and TLR4-KO mice were assessed *in vitro*. CCl₄ produced similar fibrosis and necroinflammation and increased the mRNA and protein expression of cytokines and chemokines in WT and TLR4-KO mice. However, circulating LPS concentration did not increase in CCl₄-treated mice. Interestingly, LPS down-modulated α -smooth muscle actin (activated HSC marker) and collagen 1 in both WT and TLR4-KO HSCs. LPS induced similar activation of NF- κ B, and stimulated the expression of cytokines and chemokines in WT and TLR4-KO HSCs. Finally, LPS caused similar inflammation and injury in previously untreated WT and TLR4-KO mice. The results provide evidence of the TLR4/LPS-independent mechanisms of liver fibrosis and also indicate that TLR4 is not entirely critical to LPS-induced acute liver injury. The results further indicate that LPS signaling in activated HSCs might be a mechanism of limiting liver fibrosis. (*Am J Pathol* 2017, 187: 1356–1367; <http://dx.doi.org/10.1016/j.ajpath.2017.01.021>)

Physiologically, the liver is exposed to very low concentrations of gut-derived lipopolysaccharide (LPS), a component of the outer wall of the gram-negative bacteria. LPS is efficiently cleared by the liver, but its blood levels increase during liver injury or on increased gut permeability.^{1–3} At elevated levels, LPS provokes inflammation and innate immune responses that are implicated in acute and chronic hepatic pathogenesis.^{4,5} LPS binds to circulating LPS-binding protein (LBP) and soluble or membrane CD14 that lacks cytoplasmic domain; the ternary complex binds to Toll-like receptor 4 (TLR4) on the surfaces of various cell types, causing receptor dimerization and activation.⁶ LPS activates multiple signaling pathways, including NF- κ B, nonreceptor tyrosine kinases, protein kinase C, and

mitogen-activated protein kinases in various cell types, including monocytes, macrophages, and dendritic cells,^{7–10} and induces secretion of several cytokines and chemokines, nitric oxide (NO), reactive oxygen species (ROS), and eicosanoids.^{11–13} The released mediators, including tumor necrosis factor (TNF)- α , IL-1 β , interferon (IFN)- β , IFN- γ , and ROS, create a hepatic microenvironment that is damaging to hepatocytes.^{4,5,11,13}

Experiments with CD14-overexpressing,¹² CD14-deficient,¹⁴ TLR4-mutant C57/HeJ,¹⁵ and LBP-deficient

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mice³ provided evidence for the role of CD14-LBP/TLR4 in the *in vivo* responses to endotoxin. However, LPS also causes liver injury in a CD14-independent manner.¹⁴ Furthermore, LPS-induced production of TNF- α and IL-6 in TLR4-knockout (TLR4-KO) and CD14-KO mice was found to be similar to that in wild-type (WT) mice after partial hepatectomy,¹⁶ and whereas bile duct ligation elicited similar liver injury in WT and TLR4-mutant (C3H/HeJ) mice, fibrosis was strongly reduced in the latter.¹⁷ Because TLR4 is essential for LPS actions on Kupffer cells and endothelial cells, TLR4-independent effects of LPS on other cell types are obviously important in hepatic pathophysiology.

During the last 15 years, perisinusoidal hepatic stellate cells (HSCs) have been investigated to assess their responses to LPS in hepatic inflammation, immune regulation, and fibrosis.^{18,19} HSCs are quiescent physiologically and differentiate into an activated proliferating and fibrogenic myofibroblast-like phenotype during liver injury. LPS-stimulated quiescent and activated HSCs produce many cytokines and chemokines and influence the hepatic microenvironment.^{19,20} Although the CD14-LBP/TLR4 axis can be an important mechanism of the LPS effects on HSCs, rat quiescent HSCs that express very low levels of TLR4 respond to LPS concentration as low as 1 ng/mL in serum-free medium (ie, in the absence of LBP) and produce TNF- α , IL-6, and NO.²¹ On activation, rat HSCs express increased levels of TLR4²¹ and respond to LPS with robust time-dependent increases in numerous cytokines, chemokines, adhesion molecules, and molecules associated with hepatic fibrosis in serum (LBP)-free condition.^{21–23} Human quiescent HSCs also express low levels of TLR4 and increase its expression on activation,²⁴ but mouse quiescent and activated HSCs express significantly higher TLR4.¹⁷ Thus, species-specific differences may have important implications in hepatic pathophysiology in which LPS contributes to acute and chronic liver injury. To address the TLR4-independent LPS effects, we determined carbon tetrachloride (CCl₄)-induced liver fibrosis in TLR4-KO and paired WT mice and examined effects of LPS on HSCs isolated from the WT and TLR4-KO mice. CCl₄-induced liver fibrosis was similar in WT and TLR4-KO mice, and LPS stimulated expression of cytokines and chemokines in both WT and TLR4-KO HSCs but down-regulated fibrogenic activity in them. The data indicate that TLR4-independent effects of LPS on HSCs are critical components of chronic liver injury.

Materials and Methods

Animals

B6.B10ScN-Tlr4^{lps-del}/JthJ (TLR4-KO) and their control C57BL/6J (B6-WT) mice were purchased from the Jackson ImmunoResearch Laboratories (West Grove, PA), bred in the Laboratory Animal Facility at University of Cincinnati,

and used at 8 to 10 weeks. All studies were approved by the Institutional Animal Care and Use Committee in accordance with NIH guidelines.

CCl₄ and LPS Treatments

Six mice per group for CCl₄ or LPS treatment and four mice per group as vehicle-treated controls were used. Liver fibrosis was produced by injecting 0.16 mL/kg of CCl₄ in 100 μ L of peanut oil i.p. (twice a week) for 4 weeks, and mice were euthanized at 20 to 24 hours after the last CCl₄ administration; control mice received oil alone. LPS (serotype 0111:B4; 10 mg/kg i.p.; Sigma, St. Louis, MO) or phosphate-buffered saline carrier were administered to WT and TLR4-KO mice,²⁵ which were euthanized at 6 hours. Serum was obtained from all animals, and portions of the liver were fixed in 10% neutral buffered formalin or snap frozen in liquid nitrogen.

Assessment of Liver Injury and Fibrosis

Formalin-fixed, paraffin-embedded liver sections were stained with hematoxylin and eosin for histopathologic examination, and collagen fibers were detected by staining with Sirius red in saturated picric acid as described previously.²⁶ Neutrophil staining was performed on paraffin sections using an anti-neutrophil antibody (clone NIMP-R14; Abcam, Cambridge, MA). Quantitative assessment or scoring of the hematoxylin and eosin sections for hepatic inflammation and necro-inflammation (scale of 0 to 4) and fibrosis (scale of 0 to 6) was performed according to the Ishak System²⁷ independently by two investigators (J.W. and S.K.S.) who were blinded to the samples. Serum alanine aminotransferase was measured using a kit from Catachem Inc. (Oxford, CT). Hepatic hydroxyproline was measured as described previously.²⁶

Immunostaining

Formalin-fixed liver sections (5 μ m) were deparaffinized with xylene and rehydrated through a series of graded alcohol treatment, then washed with phosphate-buffered saline. The sections were then treated overnight with anti-desmin antibody (at 1:500 dilution) (Abcam, Cambridge, MA) at 4°C. Alexa Fluor 594-conjugated goat anti-rabbit IgG secondary antibody (Thermo Fisher Scientific Inc., Waltham, MA) was used for detection and Vectashield mounting medium with DAPI for nuclear staining (Vector Laboratories, Burlingame, CA). A Zeiss Axioplan-2 imaging microscope was used to obtain the images.

Isolation and Culture of HSCs

HSCs were isolated as described previously.²⁸ Briefly, livers were digested by perfusion with Hanks' balanced salt solution containing 816 μ M CaCl₂·2H₂O, 0.25 mg/mL of

collagenase 4 (Worthington, Lakewood, NJ), and 0.50 mg/mL of protease (Sigma), excised, washed, and minced in Hanks' balanced salt solution. After filtration through a 100- μ m nylon filter, the suspension was centrifuged several times ($50 \times g$ for 2 minutes at 4°C) to remove hepatocytes and debris. After centrifugation at $600 \times g$ for 7 minutes, the nonparenchymal cell pellet was washed and HSCs purified via Histodenz (Sigma) density gradient centrifugation. HSCs were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% horse serum and seeded in 6- or 24-well plates at a density of $0.5 \times 10^6/\text{cm}^2$. Purity of the culture was >95% to 98% as determined by desmin and glial fibrillary acidic protein immunostaining (Supplemental Figure S1A), as well as vitamin A autofluorescence and flow cytometry.^{25,28,29} HSCs were used on days 5 to 7 of culture interval, and each experiment was performed in duplicate or triplicate using two to three independent cell preparations.

mRNA Analysis

RNA was extracted using Trizol Reagent (Life Technology, Carlsbad, CA) according to the manufacturer's protocol. After quantification, 1.5 μ g of RNA was reverse transcribed into cDNA using a kit from Applied Biosystems (Foster City, CA). The expression of various genes was determined using primers (Integrated DNA Technology) (Table 1) and SYBER Green master mix in a 7300 Real Time PCR System (Applied Biosystems).

Determination of Cytokines and Chemokines

Cytokines and chemokines in the culture supernatants or liver extracts were measured using Millipore Luminex kit (Darmstadt, Germany) or enzyme-linked immunosorbent assay kits (eBioscience, Inc., San Diego, CA).

LPS Determination

LPS concentration in the serum was measured using Endpoint Chromogenic Limulus Amoebocyte Lysate test kit (Lonza, Walkersville, MD).

Western Blotting

Proteins were extracted using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Dallas, TX) supplemented with protease inhibitor cocktail, phenylmethylsulfonyl fluoride, and sodium orthovanadate. After separation via 10% SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Sigma). The membranes were then blocked, incubated with primary antibodies, washed, and treated with appropriated secondary antibodies, and proteins were identified using Amersham ECL Select reagent (GE Health Care UK Ltd., Buckinghamshire, UK).

Table 1 Quantitative PCR Mouse Primer Sequences

Name	Type	Sequence
β -actin	Forward	5'-AGAGGGAAATCGTGCGTGAC-3'
	Reverse	5'-CAATAGTGATGACCTGGCCGT-3'
BAMBI	Forward	5'-TGAAGTGACTAGCAGGGAAATG-3'
	Reverse	5'-CCTGGTCTCCACAATGACTTAC-3'
CCL2	Forward	5'-CTCACCTGCTGCTACTCATTC-3'
	Reverse	5'-ACTACAGCTTCTTTGGGACAC-3'
Caspase-11	Forward	5'-GCCACTTGCCAGGTCTACGAG-3'
	Reverse	5'-AGGCCTGCACAATGATGACTTT-3'
Collagen 1	Forward	5'-AGACCTGTGTGTTCCCTACT-3'
	Reverse	5'-GAATCCATCGGTCATGCTCTC-3'
CXCL1	Forward	5'-CTGCACCCAAACCGAAGTC-3'
	Reverse	5'-AGCTTCAGGGTCAAGGCAAG-3'
IFN- γ	Forward	5'-CTCTTCCTCATGGCTGTTTCT-3'
	Reverse	5'-TTCTTCCACATCTATGCCACTT-3'
IL-10	Forward	5'-CCCTGGGTGAGAAGCTGAAG-3'
	Reverse	5'-CACTGCCTTGCTCTTATTTTCACA-3'
IL-6	Forward	5'-CCGAGAGGAGACTTCACAG-3'
	Reverse	5'-TCCACGATTTCCAGAGAAC-3'
MMP-9	Forward	5'-TGTTCCCGTTCATCTTTGAG-3'
	Reverse	5'-ATCCTGGTCATAGTTGGCTGT-3'
MMP-13	Forward	5'-GACACAGCAAGCCAGAATAAAG-3'
	Reverse	5'-GGAAAGCAGAGAGGGATTAACA-3'
TGF- β 1	Forward	5'-GGTGGTATACTGAGACACCTTG-3'
	Reverse	5'-CCCAAGGAAAGGTAGGTGATAG-3'
TGF β R1	Forward	5'-CCTTGAGTCACTGGGTGTTATG-3'
	Reverse	5'-CCACTTAGCTGCACCCATAATC-3'
TIMP-1	Forward	5'-CGGTGGGTGGATGAGTAATG-3'
	Reverse	5'-GGCTGCACAGTGGAGAATAA-3'
TIMP-2	Forward	5'-GGAATGACATCTATGGCAACC-3'
	Reverse	5'-GGCCGTGTAGATAAACTCGAT-3'
TLR4	Forward	5'-GGGTATTTGACACCCTCCATAG-3'
	Reverse	5'-CAAGAGTGCTGAGGGAATACAG-3'
TNF- α	Forward	5'-CCCAGGTATATGGGCTCATACC-3'
	Reverse	5'-GCCGATTTGCTATCTCATACCAGG-3'

BAMBI, bone morphogenic protein and activin membrane-bound inhibitor; CCL2, chemokine (C-C motif) ligand 2; IFN- γ , interferon γ ; MMP, matrix metalloproteinase; TGF- β 1, transforming growth factor 1; TGF β R1, TGF- β 1 receptor; TIMP, tissue inhibitor of metalloproteinase; TLR4, Toll-like receptor 4; TNF- α , tumor necrosis factor- α .

Determination of MMP Activity

The hepatic proteolytic activity of matrix metalloproteinases (MMPs) was measured as described.³⁰ Briefly, 10 μ g of liver lysates were incubated with activation buffer (0.1 mol/L 4-aminophenylmercuric acetate) for 1 hour at 37°C in Thermomixer, then transferred to 96-well, black-walled, clear-bottom microplates. Sixty-five microliters of 20 μ mol/L fluorogenic MMP substrate Dnp-Pro- β -cyclohexyl-Ala-Gly-Cys(Me)-His-Ala-Lys(NMA)-NH₂ (catalog number BML-P128-0001, Enzo Life Sciences, Ann Arbor, MI) in TCNB reaction buffer (50 mmol/L Tris, pH 7.5, containing 10 mmol/L CaCl₂, 0.15 mol/L NaCl, and 0.05% Brij-35) (Bio-Rad Laboratories, Hercules, CA) was added to the wells and incubation continued for 1 hour. MMP activity was measured by monitoring fluorescence (excitation/emission = 360/455 nm) over time and expressed as arbitrary units.

Statistical Analysis

The results are presented as means $\pm$ SD. Statistical significance was derived by one-way analysis of variance (Tukey's multiple comparison test) or *t*-test. $P < 0.05$ was considered significant.

Results

Similar CCl₄-Induced Liver Fibrosis in WT and TLR4-KO Mice

We confirmed absence of TLR4 mRNA expression in the liver and HSCs from all batches of TLR4-KO mice used in this study (Supplemental Figure S1B). Histopathologic examination revealed severe hepatocyte swelling and moderate to marked lobular inflammation that was more predominant in pericentral areas in association with pericentral necrosis and abundant pigment-laden macrophages in both CCl₄-treated WT and TLR4-KO mice; the portal inflammation was mild in both groups (Figure 1A and Supplemental Figure S2A). The magnitude of inflammatory infiltration, hepatocyte ballooning degeneration, and necrosis in the CCl₄-treated TLR4-KO mice were somewhat

lower compared with the WT mice; these pathologic findings were observed primarily in the centrilobular area (Figure 1A; Supplemental Figure S2A). Consistent with these findings, serum alanine aminotransferase and necroinflammation scores were lower in magnitude in CCl₄-treated TLR4-KO mice (Figure 1, B and C). The expression of CXCL1 increased similarly in WT and TLR4-KO mice and that of chemokine (C-C motif) ligand 2 (CCL2) increased only in WT mice (Supplemental Figure S2, B and C).

In addition to being an inflammatory cytokine, TNF- α is an important mediator of the activation of HSCs from their physiologic quiescent to activated fibrogenic myofibroblast-like phenotype, the major mechanism of hepatic fibrosis of various origins.^{31–33} IFN- γ , on the other hand, is a negative regulator of liver fibrosis.^{34,35} We found similarly increased hepatic levels of TNF- α in CCl₄-treated WT and TLR4-KO mice (Figure 1D). In contrast, IFN- γ protein increased significantly in CCl₄-treated WT mice despite reduced mRNA expression but not in TLR4-KO mice (Figure 1E).

TLR4/LPS interaction is reported to be a major component of CCl₄-induced liver fibrosis.¹⁷ However, there was similar CCl₄-induced fibrosis as assessed by Sirius red

Figure 1 Hepatic injury and cytokine expression in carbon tetrachloride (CCl₄)—treated Toll-like receptor 4 knockout (TLR4-KO) mice. **A:** Hematoxylin and eosin—stained liver sections reveal marked hepatocyte swelling with predominant pericentral inflammation due to CCl₄ treatment. Serum alanine aminotransferase (ALT) (**B**) and quantification of hepatic necroinflammation (**C**). **D** and **E:** mRNA and proteins were extracted from vehicle- or CCl₄-treated wild-type (WT) and TLR4-KO mouse livers. mRNA expression and concentrations of tumor necrosis factor (TNF)- α and interferon (IFN)- γ were determined by quantitative real-time RT-PCR and enzyme-linked immunosorbent assay, respectively. Data are expressed as means $\pm$ SD (**B–E**). * $P < 0.05$, ** $P < 0.005$. Scale bar = 50 μ m (**A**).

staining (Figure 2A). Although quantification revealed a slightly lower fibrosis score (Figure 2B) and collagen 1 mRNA expression (Figure 2C) in TLR4-KO mice than WT mice, the difference was not statistically significant. However, hydroxyproline content (Figure 2D) (a measure of collagen deposition) was similar. Furthermore, expression of α -SMA, an established marker of activated HSCs, increased similarly in CCl₄-treated WT and TLR4-KO mice (Figure 2E). Immunostaining for desmin (expressed by

normal and activated HSCs) revealed similarly increased hepatic accumulation of HSCs in the CCl₄-treated WT and TLR4-KO mice (Supplemental Figure S3).

The fibrogenic cytokine transforming growth factor (TGF)- β 1, secreted in the injured liver by Kupffer cells, endothelial cell, and HSCs, has been implicated in HSC activation and subsequent liver fibrogenesis.^{17,31–33} Hepatic expression of TGF- β 1 increased in CCl₄-treated WT and TLR4-KO mice, but the expression of the TGF- β 1 receptor

Figure 2 Carbon tetrachloride (CCl₄)-induced hepatic fibrosis in Toll-like receptor 4 knockout (TLR4-KO) mice. Wild-type (WT) and TLR4-KO mice were subjected to 4 weeks of CCl₄ treatment. **A:** Sirius red-stained sections of representative vehicle and CCl₄-treated livers had similarly extensive bridging fibrosis in WT and TLR4-KO mice. **B:** Hepatic fibrosis as quantified by 0 to 6 Ishak Scoring System. **C:** Collagen 1a1 (Col1a1) mRNA expression. **D:** Hydroxyproline concentration. **E:** Western blot reveals hepatic α -smooth muscle actin (α -SMA) expression in vehicle- and CCl₄-treated WT and TLR4-KO mice; bar graph shows densitometric quantification of α -SMA relative to β -actin (both antibodies from Sigma, St. Louis, MO). **F–H:** Total mRNA was extracted from vehicle- or CCl₄-treated WT and TLR4-KO mouse livers. Bar graphs show quantitative real-time RT-PCR data for the mRNA expression of transforming growth factor (TGF)- β 1, TGF- β 1 receptor (TGF β R1), and bone morphogenic protein and activin membrane-bound inhibitor (BAMBI). Data are expressed as means $\pm$ SD (**B–D, F–H**). * P < 0.05, *** P < 0.005. Scale bar = 100 μ m (**A**).

Figure 3 Lipopolysaccharide (LPS) stimulation of hepatic stellate cells (HSCs) attenuates their fibrogenic markers. HSCs isolated from wild-type (WT) and Toll-like receptor 4 knockout (TLR4-KO) mice were treated with vehicle or 100 ng/mL of LPS for 24 hours. **A–D**: mRNA expression of the proteins associated with HSC activation [α -smooth muscle actin (α -SMA) or Acta2], and fibrogenic activity [collagen 1a1 (Col1a1), transforming growth factor (TGF)- β 1 receptor (TGF β R1), and bone morphogenic protein and activin membrane-bound inhibitor (BAMBI)]. **E**: Expression of TGF- β 1 mRNA and protein release by LPS-stimulated HSCs. Data are expressed as means $\pm$ SD. * P < 0.05.

(TGF β R1) increased only in the WT mice (Figure 2, F and G). Interestingly, hepatic mRNA expression of the TGF- β pseudoreceptor bone morphogenic protein and activin membrane-bound inhibitor (BAMBI) was similar in control and CCl₄-treated WT mice but decreased in TLR4-KO mice (Figure 2H). Because BAMBI is down-regulated by LPS,¹⁷ we determined its hepatic expression and that of TGF- β 1 and TGF β R1 in LPS-treated mice and found that their expressions were lower in LPS-treated WT and TLR4-KO mice, the effect being much stronger for BAMBI (Supplemental Figure S4A). We reasoned that if LPS plays a major role in hepatic fibrosis, its circulating concentration should be elevated. However, the serum concentration of LPS in both CCl₄-treated WT and TLR4-KO mice was essentially similar to the control levels (Supplemental Figure S4B). To examine whether TLR4 is activated in CCl₄-treated mice, we determined the expression of TLR4 and MyD88 via Western analysis and compared that with the expression level in LPS-treated mice. LPS treatment increased TLR4 expression but not of MyD88; in contrast, CCl₄ treatment increased the expression of both TLR4 and MyD88 (Supplemental Figure S5A).

Because noncanonical activation of inflammasome, especially caspase-11, is reported to mediate TLR4-independent effects of LPS,³⁶ we examined hepatic caspase-11 expression in WT and TLR4-KO mice after LPS or CCl₄ treatments.

Although caspase-11 expression increased strongly in LPS-treated mice, no such effect was observed in CCl₄-treated WT or TLR4-KO mice (Supplemental Figure S5B). These data suggest that although both TLR4-dependent and TLR4-independent pathways are involved in LPS-induced acute liver injury, LPS may not be critical to fibrosis development.

The net deposition of extracellular matrix is regulated by MMPs and tissue inhibitors of metalloproteinases (TIMPs). MMP13 (MMP1 in human) and MMP9 degrade collagens, whereas TIMP1 and TIMP2 inhibit the activities of the MMPs.³⁷ We observed a significant increase in the expression of MMP9, MMP13, and TIMP1 in CCl₄-treated WT mice but not in TLR4-KO mice; TIMP2 expression increased modestly in CCl₄-treated WT and TLR4-KO mice, but the increase was not significant statistically (Supplemental Figure S6, A and B). The results suggested that unaltered expression of MMPs might be an important factor in fibrosis in TLR4-KO mice. Indeed, measurement of the enzymatic activity *in vitro* revealed no significant difference between vehicle and CCl₄-treated mice (Supplemental Figure S6C). Because the gene expression of MMPs is regulated at the transcriptional level, activation of latent proenzyme by proteolytic cleavage, and inhibition by TIMPs,³⁷ the data suggest that compromised cleavage of pro-MMPs and/or inhibition of MMP activity by contemporaneously increased TIMP1 *in vivo* may result in increased extracellular matrix deposition

in CCl₄-treated mice. Alternatively or additionally, the synthesis or deposition of the extracellular matrix is sufficiently excessive to allow significant MMP-induced degradation to be apparent.

LPS Down-Regulates α -SMA and Collagen 1 Expression in Activated HSCs from WT and TLR4-KO Mice

We previously reported that rat HSCs increase TLR4 expression on activation²¹ and LPS down-regulates expression of α -SMA and collagen 1 in activated rat HSCs.²³ Considering similar CCl₄-induced fibrosis in WT and TLR4-KO mice (Figure 2), we examined the effects of LPS in culture-activated WT and TLR4-KO HSCs. LPS caused reduction in the mRNA expression of *Acta2* (gene that encodes α -SMA) and *Colla1* in both WT and TLR4-KO HSCs (Figure 3, A and B). LPS down-regulated mRNA expression of TGF β R1, but not of BAMBI, in WT HSCs (Figure 3, C and D); LPS did not significantly reduce the mRNA expression of TGF β R1 or BAMBI in TLR4-KO HSCs, although the trend appears similar to that for WT HSCs. We also found that LPS did not significantly alter mRNA expression or release of TGF- β 1 by WT HSCs; in contrast, TGF- β 1 mRNA expression and protein release were relatively greater in TLR4-KO HSCs, and whereas LPS induced a insignificant decrease in mRNA expression of TGF- β 1 in TLR4-KO HSCs, the protein release was significantly reduced (Figure 3E).

Because LPS-induced down-regulation of BAMBI in quiescent HSCs occurred at 6 hours,¹⁷ a possibility that BAMBI may be down-regulated at an earlier time during LPS challenge was examined. In culture-activated WT or TLR4-KO HSCs, LPS did not down-regulate BAMBI at 6 hours (Supplemental Figure S7A). These findings and down-regulation of BAMBI in LPS-treated normal mice at 6 hours suggest that the effect may be distinct in quiescent and activated HSCs. We examined a possibility that TGF- β 1 may reverse the inhibitory effect of LPS on α -SMA and collagen 1 expression in activated HSCs. However, TGF- β 1-induced increase in α -SMA and collagen 1 expression were inhibited in presence of LPS (Supplemental Figure S7B). Furthermore, TGF- β 1 slightly increased BAMBI expression in WT HSCs, but this effect was not apparent in the presence of LPS (Supplemental Figure S7A).

Cytokine and Chemokine Expression by LPS-Stimulated TLR4-KO HSCs

LPS-induced strong increase in the mRNA expression of TNF- α and IL-10 in WT HSCs; in TLR4-KO HSCs, however, LPS modestly increased the expression of TNF- α and IL-10 (Figure 4, A and B). The LPS-induced mRNA expression of the cytokines was consistent with similar increases in the corresponding proteins in culture supernatants of LPS-stimulated WT or TLR4-KO HSCs (Figure 4, A and B). No expression of IFN- γ , IL-2, IL-13, and IL-17A was detected in

Figure 4 Lipopolysaccharide (LPS)–induced expression of cytokines and chemokines in Toll-like receptor 4 knockout (TLR4-KO) hepatic stellate cells (HSCs). HSCs were incubated in a medium that contained vehicle (grey bars) or 100 ng/mL of LPS (black bars) for 6 hours (left panels) or 24 hours (right panels). The mRNA expression of the cytokines (A and B) and chemokines (C and D) was measured by real-time quantitative PCR, and cytokines and chemokines released in the culture supernatants as measured by Luminex assay. Data are expressed as means $\pm$ SD. * P < 0.05. KC, keratinocyte chemoattractant; MCP-1, macrophage chemotactic protein-1.

unstimulated or LPS-stimulated WT or TLR4-KO HSCs (data not shown). Because contaminating factors in LPS might mediate effects via other TLRs, such as TLR2, HSCs were stimulated with lipid A, the highly conserved component of LPS. Lipid A was found to produce equivalent effect as LPS on WT and TLR4-KO HSCs (Supplemental Figure S8).

In addition to the cytokines of hepatic injury and protection, LPS also stimulates the expression of several chemokines by mouse^{17,28} and rat HSCs.²³ We examined the TLR4 dependence of LPS-induced expression and release of neutrophil-, monocyte-, and T-cell-attractant KC

Figure 5 Lipopolysaccharide (LPS)–induced activation of NF-κB and expression of tumor necrosis factor (TNF)-α, IL-10, and CXCL1 in Toll-like receptor 4 knockout (TLR4-KO) hepatic stellate cells (HSCs). **A:** HSCs were incubated in a medium that contained vehicle or 100 ng/mL LPS for 15 minutes. Western blot reveals phospho-IκBα (P-IκBα), phospho-NF-κB-p65 (P-p65), and β-actin (to ensure equal loading); bar graph shows densitometric quantification. **B:** HSCs were treated with 10 μmol/L NF-κB inhibitor (NFκB-i) BMS-345541 for 1 hour. The cells were then stimulated with 100 ng/mL of LPS for 6 hours. The bar graphs show mRNAs expression of TNF-α, IL-10, and CXCL1 as measured via quantitative real-time RT-PCR. Data are expressed as means ± SD. **P* < 0.05, ***P* < 0.005, ****P* < 0.0005.

(keratinocyte chemoattractant encoded by *Cxcl1*) and monocyte-, macrophage-, dendritic cell-, T-cell–attractant macrophage chemotactic protein-1 encoded by *Ccl2*. LPS increased the mRNA expression of CXCL1 and CCL2 by WT HSCs; the effect of LPS on TLR4-KO HSCs was almost similar for CXCL1 and modest for CCL2 in comparison to WT HSCs (Figure 4, C and D). The secretion of corresponding chemokines was consistent with the changes in mRNA expression (Figure 4, C and D).

LPS-Induced NFκB Activation and Its Coupling to Cytokine/Chemokine Expression in TLR4-KO HSCs

NFκB mediates LPS-induced synthesis of inflammatory cytokines in HSCs.^{23,24,38} Activation of NFκB involves phosphorylation-induced and proteasome-mediated degradation of the associated inhibitory component IκB; phosphorylated NFκB then translocates to nucleus and stimulates transcription of numerous cytokines, chemokines, and growth mediators.³⁹ We observed constitutively expressed phosphorylated NFκB-p65 in WT and TLR4-KO HSCs, and LPS stimulation of both HSC phenotypes resulted in robust activation of NFκB accompanied by phosphorylation of IκBα (Figure 5A). We then assessed the effect of NFκB blockade on the key molecules in HSCs. Blocking NFκB caused inhibition of the expression of TNF-α, IL-10, and CXCL1 both in WT and TLR4-KO HSCs (Figure 5B). Together, the results indicate that TLR4 is not essential for LPS-induced NFκB activation or the synthesis of TNF-α, IL-10, or KC in activated mouse HSCs.

TLR4-Independence of LPS-Induced Liver Injury

We recently reported a critical role of HSCs in LPS-induced hepatic inflammation and injury.²⁵ On examining the role of TLR4, the livers of LPS-treated WT and TLR4-KO mice showed similar histological changes (Figure 6A), including mild hepatocyte swelling, and mild lobular and portal inflammation (Figure 6A; Supplemental Figure S9). The histological observations were consistent with similar necro-inflammatory scores (Figure 6B), and serum alanine aminotransferase levels in LPS-treated WT and TLR4-KO mice (Figure 6C). The expression of CCL2 increased similarly in LPS-challenged WT and TLR4-KO mice, and the increase in CXCL1 was significant but of lower magnitude in TLR4-KO mice compared to WT mice (Figure 6, D and E).

Hepatic mRNA expression of TNF-α, IFN-γ, and IL-10 all showed strong increase in LPS-treated WT mice, and relatively small but significant increase in LPS-treated TLR4-KO mice (Figure 7A). The increase in the protein levels of these cytokines was consistent with the increase in mRNA expression, with the exception of TNF-α in LPS-treated TLR4-KO mice (Figure 7B). The basal hepatic concentrations of IFN-γ, and especially IL-10 were already somewhat higher in TLR4-KO mice compared to the WT mice.

Discussion

LPS-induced signaling through LBP-CD14/TLR4 plays crucial role in acute liver injury and fibrosis, and LPS-HSC

Figure 6 Lipopolysaccharide (LPS)-induced hepatic inflammation, injury, and chemokine expression in Toll-like receptor 4 knockout (TLR4-KO) mice. Wild-type (WT) and TLR4-KO mice were administered vehicle or 10 mg/kg of LPS and sacrificed at 6 hours. **A:** Hematoxylin and eosin-stained sections showing mild lobular and portal inflammation in LPS-treated WT and TLR4-KO mice. **Arrows** indicate focal lobular inflammation; **right panel** shows inflammation in high-magnification images of LPS-treated mouse livers. **B:** Necroinflammatory scores as quantified according to Ishak Scoring System. **C:** Serum alanine aminotransferase (ALT) levels. **D and E:** Hepatic mRNA expression of chemokine (C-C motif) ligand (CCL2) and CXCL1 as determined by quantitative real-time RT-PCR. Data are expressed as means $\pm$ SD (**B–E**). * $P < 0.05$, ** $P < 0.005$. Scale bar: 50 μ m (**left and middle panels**); 20 μ m (**right panels**).

interactions can be important components of these pathologies. Here we found that TLR4-independent signaling may also be critical in CCl_4 -induced hepatic fibrosis and LPS-induced acute injury in mice. Although LPS stimulated the expression/release of $\text{TNF-}\alpha$, IL-10, KC, and macrophage chemotactic protein-1 in TLR4-KO HSCs, the magnitude of the effect was somewhat lower than in WT HSCs, indicating the role of TLR4-dependent and -independent mechanisms. LPS also induced NF- κ B activation similarly in WT and TLR4-KO HSCs, and its blockade caused inhibition of the cytokine and chemokine expression. These effects of LPS on HSCs are interesting because the macrophages obtained from *Tlr2*-, *Tlr4*-, *Cd14*-, and *Myd88*-KO mice do not produce TNF in response to LPS, and LPS does not increase circulating levels of the cytokine.^{14,40,41}

If TLR4 is absolutely essential for hepatic effects of LPS *in vivo*, TLR4-KO mice should be resistant to hepatic inflammation and injury, given that LPS is a very potent inflammatory mediator. Thus, unlike the WT mice, TLR4-mutant C3H/HeJ mice did not have acute-phase protein response on LPS challenge; this effect was interpreted to be due to the inability of C3H/HeJ mice to produce significant levels of proinflammatory cytokines.^{14,42} However, we observed similar level of inflammatory infiltration, necroinflammation, and liver injury in LPS-treated WT and the

homozygous TLR4-KO B6.B10ScN-Tlr4^{lps-del}/JthJ mice and relatively lower (but significant) increases in hepatic expression of $\text{TNF-}\alpha$ and CXCL1 in LPS-treated TLR4-KO mice. These findings are similar to the direct effect of LPS on WT and TLR4-KO HSCs *in vitro*. Clearly, the comparatively lower hepatic increase in the inflammatory mediators in TLR4-KO mice appears sufficient to induce similar injury as in WT mice.

CCl_4 -induced liver fibrosis was found to be strongly reduced in TLR4-mutant C3H/HeJ mice compared with WT mice.¹⁷ In contrast, CCl_4 -induced fibrosis in WT and TLR4-KO B6.B10ScN-Tlr4^{lps-del}/JthJ mice was similar as determined by fibrosis score, collagen 1 expression, and hydroxyproline content. Furthermore, α -SMA expression (an established marker for activated HSCs, the major fibrogenic cell in the liver) and accumulation of desmin-positive cells (ie, HSCs) increased similarly in CCl_4 -treated WT and TLR4-KO mice. TGF- β 1, the most potent fibrogenic cytokine, is produced at low levels in physiology by endothelial cells and Kupffer cells and mainly by HSCs during inflammation and fibrosis.⁴³ TGF- β 1 expression increased in both WT and TLR4-KO mice, but the expression of TGF β RI increased only in WT mice. $\text{TNF-}\alpha$ -induced increased surface kallikrein causes the release of TGF- β 1 in its active form by activated HSCs.⁴⁴ Previous work found that stimulation with bacterial cell wall products lipoteichoic

Figure 7 Lipopolysaccharide (LPS)-induced hepatic expression of cytokines in Toll-like receptor 4 knockout (TLR4-KO) mice. Wild-type (WT) and TLR4-KO mice were administered vehicle (grey bars) or 10 mg/kg LPS (black bars) and sacrificed at 6 hours. Hepatic mRNA (A) and protein (B) levels of tumor necrosis factor (TNF)- α , interferon (IFN)- γ , and IL-10 were measured by quantitative real-time RT-PCR and enzyme-linked immunosorbent assay, respectively. Data are expressed as means $\pm$ SD. * P < 0.05, ** P < 0.005, *** P < 0.0005.

acid or *N*-acetyl muramyl peptide, but not LPS, induced significant increase in TGF- β 1 expression and release by BALB/c HSCs.⁴⁵ Although the increase in hepatic TGF- β 1 expression in CCl₄-treated TLR4-KO mice was lower than in WT mice, it is plausible that most of the cytokine may be released in the active form and thus induces fibrogenic activity in HSCs.

It was suggested that LPS-induced down-regulation of BAMBI is critical to the activation and fibrogenic effect of TGF- β 1 on HSCs.¹⁷ However, hepatic BAMBI expression was essentially unaltered in CCl₄-treated WT and TLR4-KO mice. To gain an insight into these contrasting observations, we determined the effect of LPS on TGF- β 1, TGF β R1, and BAMBI expression in previously normal mice. Interestingly, TGF- β 1 and TGF β R1 expression decreased in LPS-treated WT and TLR4-KO mice, but the down-regulation of BAMBI expression was much stronger. In contrast, LPS did not affect BAMBI expression in culture-activated mouse HSCs (Figure 3D; Supplemental Figure S7A) and actually increased its expression in culture-activated rat HSCs.²³ Furthermore, serum LPS levels were similar in WT and TLR4-KO mice and did not alter significantly after CCl₄ treatment. These findings suggest that LPS may not regulate BAMBI expression during fibrosis progression. However, the possibility that during the early phase of acute liver injury a sudden increase in LPS in the portal circulation may transiently down-regulate BAMBI in HSCs (as seen with acute LPS treatment) (Supplemental Figure S4), thus allowing TGF- β 1 to promote their activation and fibrogenic function in conjunction with

inflammatory mediators, cannot be ruled out. It is worth noting that although long-term alcohol ingestion causes increase in LPS and TNF- α in rats and mice, the animals develop steatosis and steatohepatitis but not fibrosis.^{46–48}

Free radicals, including CCl₄-derived CCl₃·, are considered to be primary stimuli of hepatocyte death.^{49,50} The acute phase of CCl₄-mediated liver fibrosis is characterized by activation of Kupffer cells, which can occur on engulfment of apoptotic bodies derived from dying hepatocytes.⁵¹ CCl₄ thus elicits an inflammatory response, resulting in secretion of cytokines, chemokines, and other proinflammatory factors that attract and activate monocytes, neutrophils, and lymphocytes, which further contribute to liver cell death.^{49,50} Apoptotic bodies⁵² and free radicals, as well as inflammatory mediators such as TNF- α , are also strong stimuli for HSC activation.^{31,32} Thus, it can be concluded that TLR4-independent release of mediators from Kupffer cells and HSCs themselves are critically important in fibrosis development and an important mechanism of fibrosis in both WT and TLR4-KO mice.

The differential effects of CCl₄ in causing fibrosis in C3H/HeJ¹⁷ and B6.B10ScN-Tlr4^{Alps-del/JthJ} (TLR4-KO) mice used in this investigation might be explained by the mode of the toxin administration. For example, C57BL/6J mice were found to be resistant to fibrosis when CCl₄ was administered via gavage⁵³ but not when administered i.p.⁵⁴ It is possible that CCl₄ administration by gavage or toxicity of thioacetamide metabolite derived from the effect of cytochrome P450 may increase gut permeability^{55,56} and/or LPS release, resulting in its leakage into the portal blood.¹⁷

In addition, the bile duct ligation model used by Seki et al¹⁷ certainly disrupts gut homeostasis through the loss of bile acids in the gut, allowing LPS translocation.^{57,58} Furthermore, Hillebrandt et al⁵⁴ observed similar increase in hydroxyproline levels at 6 weeks of CCl₄ administration in C3H/HeJ and BALB/c mice and intermediate increase in C57BL/6J mice. It will be of interest to determine what type(s) of compensatory mechanisms are instigated as a result of TLR4 mutation (C3H/HeJ) versus TLR4 deletion (B6.B10ScN-Tlr4^{lps-del}/JthJ) to understand the discrepancy in distinct response to fibrogenic stimulation.

IFN- γ inhibits HSC activation and fibrogenesis,^{34,35} and CCl₄-induced (gavage) fibrosis was more pronounced in mice lacking IFN- γ than the WT mice.⁵³ The net outcome of a pathologic process is determined by the balance of activity between the proinflammatory and anti-inflammatory factors. Thus, it is reasonable to postulate that an increase in hepatic IFN- γ on treatment of WT mice with CCl₄ (Figure 1) is an intrinsic mechanism of resisting fibrosis. The inability of the TLR4-KO mice to increase hepatic IFN- γ may be interpreted as a loss of such counterregulatory mechanism of fibrosis, resulting in the elevation of fibrosis development to the WT level.

We previously found that LPS inhibits DNA synthesis in activated HSCs.^{21,22} We also observed that LPS down-regulates TGF- β 1, TGF β R1, α -SMA, and collagen 1 expression in activated rat HSCs.²³ Consistently, LPS strongly down-regulated the gene expression of *Acta2*, *Colla1*, and *Tgf β 1* not only in WT but also in TLR4-KO HSCs. These data suggest that LPS may act as a counter-regulator of fibrogenic function of HSCs.

In summary, results of the present investigation provide evidence that during persistent inflammatory injury as that with CCl₄ administrations, LPS-TLR4 interactions in activated HSCs may not be critical to hepatic fibrosis. The data, in fact, suggest that LPS may play an important role in limiting fibrosis by down-regulating TGF- β 1, TGF β R1, and α -SMA in activated HSCs.

Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2017.01.021>.

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