Inhibition of Toll-like receptor 4 suppresses liver injury induced by biliary obstruction and subsequent intraportal lipopolysaccharide injection

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Oya S, Yokoyama Y, Kokuryo T, Uno M, Yamauchi K, Nagino M. Inhibition of Toll-like receptor 4 suppresses liver injury induced by biliary obstruction and subsequent intraportal lipopolysaccharide injection. Am J Physiol Gastrointest Liver Physiol 306: G244–G252, 2014. First published December 19, 2013; doi:10.1152/ajpgi.00366.2013.—The objective of this study was to elucidate the role of Toll-like receptor 4 (TLR4) in liver injury induced by biliary obstruction and subsequent intraportal lipopolysaccharide (LPS) infusion in rats. Biliary obstruction often leads to the development of bacterial translocation. Rats were subjected to either a sham operation (Sham group) or bile duct ligation for 7 days (BDL group). Seven days after each operation, LPS (0.5 μg) was injected through the ileocecal vein. In other experiments, rats that had undergone BDL were pretreated, before LPS challenge, with internal biliary drainage (Drainage group); intravenous TAK-242, a TLR4 inhibitor (TAK group); or intravenous GdCl3, a Kupffer cell deactivator (GdCl3 group). The expression of the TLR4 protein and the number of Kupffer cells in the liver were significantly increased in the BDL group compared with the Sham group. These changes were normalized after biliary drainage. The expression of TLR4 colocalized with Kupffer cells, which was confirmed by double immunostaining. Serum levels of liver enzymes and proinflammatory cytokines after intraportal LPS injection were significantly higher in the BDL group than in the Sham group. However, pretreatment with TAK-242 or GdCl3 strongly attenuated these changes to levels similar to those seen with biliary drainage. These results imply that blocking TLR4 signaling effectively attenuates liver damage to the same level as that observed with biliary drainage in rats with BDL and subsequent intraportal LPS infusion. TAK-242 treatment may be used for patients who are susceptible to liver damage by biliary obstruction and endotoxemia.

cholangiocarcinoma or pancreatic head cancer often develop biliary obstruction. Biliary obstruction leads to impaired intestinal barrier function and the translocation of enteric bacteria to the systemic circulation (10, 26, 31). Thus, patients with biliary obstruction are susceptible to septic complications not only with cholangiovenous reflux but also with increased bacterial translocation (BT) from the gut to the systemic circulation (1, 11, 21). Severe liver damage has been shown using a model of biliary obstruction [bile duct ligation (BDL)] followed by endotoxin challenge by duodenal, intravenous, or intraperitoneal lipopolysaccharide (LPS) administration (2, 6, 18). However, no study has ever administered LPS through portal circulation (which mimics the conditions of BT) in BDL rats.

A previous report demonstrated that intraportal LPS administration induces severe liver injury via Toll-like receptor 4 (TLR4) activation on Kupffer cells and the production of excessive amounts of proinflammatory cytokines (3). However, the mechanistic role of TLR4 or Kupffer cells in liver injury induced by biliary obstruction and BT has not been fully investigated. Moreover, no effective therapy, except biliary drainage, exists to treat this detrimental condition.

TAK-242 is a small-molecule TLR4 inhibitor that interferes with signal transduction mediated through the CD14-TLR4 complex without directly inhibiting the binding of LPS to TLR4 (7, 13). Pretreatment with TAK-242 yielded a survival benefit in lethal porcine (5) and murine (24) sepsis models. However, TAK-242 has never been used in rats with BDL.

The aim of this study was to elucidate the mechanism of liver injury in a rat BDL model with subsequent intraportal LPS administration, which mimics the conditions of biliary obstruction accompanied by BT. Our hypothesis was that TLR4 in Kupffer cells may play a key role in liver damage and that the blockade of TLR4 signaling by TAK-242 may be a novel therapeutic option for such conditions.

MATERIALS AND METHODS

Chemicals. Ethyl (6R)-6-[[2-chloro-4-fluorophenyl]sulfamoyl]cyclohex-l-ene-l-carboxylate (TAK-242) was purchased from ChemScene (Monmouth Junction, NJ). Gadolinium trichloride (GdCl3) was purchased from Tocris (Bristol, UK). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO).

Animals. Male Wistar rats (Charles River Labs, Wilmington, MA) weighing 250–300 grams were purchased from Japan SLC (Nagoya, Japan) and housed in a temperature- and humidity-controlled environment on a constant 12:12-h light-dark cycle. Animals were allowed access to water and food ad libitum. All experiments were approved by the Institute for Laboratory Animal Research, Nagoya University Graduate School of Medicine.

Surgical procedures. All surgical procedures were performed under general anesthesia by inhalation of isoflurane. An abdominal midline incision was made, and the common bile duct (CBD) was ligated at two points with 5–0 silk threads and divided between them (the BDL group). However, in the Sham group, the CBD was freed from the surrounding tissues without ligation or transection.

In some of the rats, the abdomen was reopened through the previous incision 7 days after BDL, and biliary drainage was performed by inserting a silicone catheter (inside diameter = 0.5 mm, outside diameter = 1.0 mm; Imamura, Tokyo, Japan) proximally in the CBD and distally in the duodenum by applying a purse string suture with 5–0 polypropylene thread (the Drainage group). Intraportal administration of LPS. LPS (0.5 ml; purified from Escherichia coli O111:B4, 1 μg/ml) was slowly injected through the ileocecal vein via a 26-gauge needle with a micro syringe in the Sham, BDL, and Drainage groups. In the Drainage group, LPS was administered 24 h after the drainage operation. The rats were killed, and blood, urine, liver, and lung samples were harvested 0.5, 2, and 4 h after LPS injection. In some of the rats in the BDL group, TAK-242 was administered 24 h after the drainage operation.

PATIENTS WITH CHOLANGIOCARCINOMA or pancreatic head cancer often develop biliary obstruction. Biliary obstruction leads to impaired intestinal barrier function and the translocation of enteric bacteria to the systemic circulation (10, 26, 31). Thus, patients with biliary obstruction are susceptible to septic complications not only with cholangiovenous reflux but also with increased bacterial translocation (BT) from the gut to the systemic circulation (1, 11, 21). Severe liver damage has been shown using a model of biliary obstruction [bile duct ligation (BDL)] followed by endotoxin challenge by duodenal, intravenous, or intraperitoneal lipopolysaccharide (LPS) administration (2, 6, 18). However, no study has ever administered LPS through portal circulation (which mimics the conditions of BT) in BDL rats.

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(1 mg/kg iv) was administered 1 h before LPS injection (the TAK group) to block TLR4-mediated signaling, or GdCl3 (1 mg/kg iv) was administered 24 h before LPS injection to block Kupffer cell function (the GdCl3 group). Rats in those two groups were killed 2 and 4 h after LPS challenge (Fig. 1). Both TAK-242 and GdCl3 were dissolved in PBS and administered from the penile vein. The amount of PBS used to dissolve TAK-242 or GdCl3 was very small, and the results of a vehicle only group may not be different from that of the BDL group (without using vehicle). Therefore, we omitted the unnecessary control group (i.e., vehicle only group) based on an animal welfare standpoint.

Analysis of blood samples. Serum levels of endotoxin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured using standard laboratory methods (SRL, Tokyo, Japan).

Analysis of tissue samples. One hundred milligrams of liver and lung tissue were mixed with 1,000 μl PBS, homogenized, and centrifuged at 16,060 g for 3 min. Endotoxin levels in the supernatant were detected using standard laboratory methods (SRL) and were expressed as picograms per milliliter.

Histological evaluation. Liver tissue samples were fixed immediately in 10% buffered formalin, dehydrated in a graded ethanol series, embedded in paraffin, and then stained with hematoxylin and eosin. The tissue sections were examined under light microscopy for the percentage of liver necrosis in five randomly selected low-powered (×10 objective) fields of view for each rat. The examined views were recorded and analyzed using cellSens Dimension (Olympus, Tokyo, Japan). Other sections were subjected to immunohistochemistry to detect the expression of TLR4. The automated slide preparation system Discovery XT (Ventana Medical Systems, Tucson, AZ) was used. Before being stained, paraffin sections were heated at 37°C for 30 min in a paraffin oven and were blocked with 5% nonfat milk. The staining procedure was carried out according to the manufacturer’s protocol (Ventana Medical Systems). Anti-TLR4 antibody (Abcam, Cambridge, UK) was diluted in Discovery Ab diluent (Ventana Medical Systems).

The other sections were subjected to double immunostaining with anti-TLR4 (Abcam) and anti-ED-1 (an antigen expressed by Kupffer cells) antibodies (Bioss, Boston, MA). The sections were dewaxed with aliphatic hydrocarbons, gradually hydrated with gradient alcohol, and washed with PBS. The sections were blocked with 10% normal goat serum and were incubated with species-specific anti-TLR4 and anti-ED-1 primary antibodies (the anti-ED-1 antibodies were conjugated with Alexa 488 dye) at 4°C overnight. After being washed with PBS, the sections were incubated for 30 min in secondary antibody for TLR4 conjugated with Alexa 594 dye. After being washed with PBS, the sections were incubated for 5 min in DAPI (Cell Signaling Technology, Boston, MA). After being washed with PBS, the sections were mounted with aqueous mounting medium (Diagnostic BioSystems, Pleasanton, CA) under a cover slip. The sections were then examined using an LED excitation system (Cool LED pE excitation system).

Quantitative real-time PCR. To validate gene expression changes in the liver, quantitative real-time PCR analysis was performed with a Prism 7300 sequence detection system (Applied Biosystems, Foster City, CA) (12). Total RNA was isolated from whole liver using the QIAcube (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. cDNA was generated from total RNA samples using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each reaction was performed in a 20-μl mixture, which included TaqMan universal PCR master mix according to the manufacturer’s instructions (Applied Biosystems). Expression of the gene encoding the LPS receptor [TLR4 (assay identification no. Rn00569848_m1; Applied Biosystems)] in the liver homogenate was determined by comparative quantitative real-time PCR using the Prism 7300 sequence detection system. 18S rRNA (assay identification no. Hs99999901_s1; Applied Biosystems) was used as an endogenous control. All samples were tested in duplicate.

Enzyme-linked immunosorbent assay. TNF-α and IL-6 levels in the serum were determined using sandwich enzyme-linked immunosorbent assay (ELISA) methods. The assay was performed according to the manufacturer’s protocol (R&D, Minneapolis, MN).

Western blot analysis. The liver samples were homogenized in Laemmli sample buffer. They were electrophoresed on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Billerica, MA). Anti-TLR4 antibodies (Abcam) were detected using an ECL system (GE healthcare Life Science, Buckinghamshire, UK) and exposed to X-ray film (Fuji Photo Film, Tokyo, Japan) (28). The average intensities of the resultant band were analyzed by densitometry using the free online software, ImageJ.

In vivo microscopy. The animals were anesthetized with pentobarbital sodium (50 mg/kg body wt ip). Latex beads (2 μm in diameter; Sigma-Aldrich) were injected through the carotid artery. Ten minutes after latex bead injection, the left lateral lobe of the liver surface was epi-illuminated with an LED excitation system (Cool LED pE excitation system) using 330–385 nm excitation and >420 nm emission bandpass filters to visualize latex beads in the sinusoids. The images were recorded using a C10600 ORCA-R2 Digital Camera and processed using Aquacosmos software (Hamamatsu Photonics, Hamamatsu, Japan). In all experiments, four fields consisting of four to eight acini per field were randomly recorded using a ×20 objective. The average number of adherent latex beads, which were taken up specifically by Kupffer cells, in five fields for each rat (n = 3 in the Sham, BDL, and Drainage groups) was determined during playback of videotaped images.
Statistical analysis. Student’s t-test was used to compare significant differences between two groups. Significant differences among multiple groups were analyzed using a one-way ANOVA followed by the Dunnett’s test. When criteria for parametric testing were violated, the appropriate nonparametric Mann-Whitney U-test was used. A P value of <0.05 was considered significant. All results are presented as means ± SE.

RESULTS

TLR4 expression in the liver. In the BDL group, expression of TLR4 mRNA in the liver was significantly higher compared with the Sham group. The increased expression of the TLR4 gene was significantly attenuated 24 h after biliary drainage in

Fig. 2. A: gene expression of Toll-like receptor 4 (TLR4) in the liver in the Sham, BDL, and Drainage groups detected using real-time RT-PCR. *P < 0.05 vs. Sham, †P < 0.05 vs. BDL. N = 6 in each group. B: Western blot images for TLR4 and β-actin (internal control). C: expression of the TLR4 protein in the liver in the Sham, BDL, and Drainage groups analyzed by densitometry. The intensity of TLR4 in each sample was divided by that of the corresponding β-actin. *P < 0.05 vs. Sham, †P < 0.05 vs. BDL. N = 4 in each group. D: immunohistochemistry images for TLR4 protein in the Sham, BDL, and Drainage groups. The images were recorded using a ×40 objective.
the Drainage group (Fig. 2A). Levels of TLR4 protein among the three groups showed a trend similar to that of mRNA expression (Fig. 2, B and C). Immunostaining revealed strong TLR4 expression along the sinusoid in the BDL group, which was distinct from that in the Sham and Drainage groups (Fig. 2D).

The number of latex beads engulfing cells. The number of cells that engulfed latex beads was counted using in vivo microscopy. Although the latex beads may be engulfed by the infiltration macrophages and certain portion of neutrophil, the location of beads detected by in vivo microscopy indicated that the major cells engulfing latex beads were Kupffer cells (the residential macrophages). In the BDL group, the number of latex beads engulfing cells was significantly increased compared with the Sham group (Fig. 2). This change in the BDL rat was normalized in the Drainage group.

Double immunostaining. A double immunostaining with anti-TLR4 and anti-ED-1 antibodies revealed a colocalization of these two antigens, indicating that TLR4 was mainly expressed in Kupffer cells (Fig. 4). Additionally, the expressions of TLR4 and ED-1 in the BDL group were much higher compared with those in the Sham and Drainage groups.

Endotoxin levels in serum, urine, liver, and lung. The serum endotoxin level was markedly increased after intraportal LPS administration both in the Sham and BDL groups (Fig. 5). The levels tended to be lower in the BDL group than the Sham group and gradually decreased over the time course in both groups. Endotoxin was almost undetectable in the urine in both groups. In the tissue samples, a high level of endotoxin was detected in the liver after intraportal LPS administration. The time-dependent change in liver tissue LPS concentration was parallel with that of the serum. However, endotoxin was almost undetectable in the lung. These results indicate that the liver may be a major organ that captures endotoxin entering the portal circulation.

Histological evaluation. Liver samples from the Sham, BDL, and Drainage groups before and 4 h after LPS administration were examined histologically (Fig. 6). The percentage of area that was necrosed was significantly increased in the BDL group 4 h after LPS injection compared with the Sham group and showed a significant decrease in the Drainage group compared with the BDL group. The necrotic area in the BDL group was mainly observed in the periportal area (Zone 1).

Administration of TAK-242 and GdCl3. Under the hypothesis that overactivation of TLR4 in Kupffer cells is a major factor that is responsible for liver damage under conditions of BDL and subsequent intraportal LPS administration, we used either a TLR4 inhibitor (TAK-242) or a Kupffer cell deac-
Activator (GdCl₃) before LPS challenge. Serum AST and ALT levels 4 h after LPS injection were significantly higher in the BDL group compared with the Sham or Drainage groups (Fig. 7, A and B). Interestingly, both TAK-242 and GdCl₃ pretreatment significantly attenuated the elevation of AST and ALT after LPS injection. The levels of these enzymes were almost identical to those in the Drainage group. The levels of plasma TNF-α and IL-6 were also markedly increased 2 and 4 h after LPS injection in the BDL group. However, these changes were significantly attenuated in the TAK and GdCl₃ groups (Fig. 7, C and D).

Mortality study. Although all rats survived until 8 h after LPS injection, 50% of the rats in the BDL group did not survive more than 24 h. Treatment with biliary drainage,
TAK-242, and GdCl₃ improved the survival rate 24 h after LPS challenge to 92, 100, and 83%, respectively.

**DISCUSSION**

Biliary obstruction promotes intestinal barrier dysfunction and predisposes for the occurrence of BT (1). Patients with biliary tract cancer or pancreatic head cancer frequently have obstructive jaundice, and these patients need to undergo highly invasive surgeries, such as major hepatectomies or pancreatoduodenectomies. We previously demonstrated a high rate of BT (~37%) in mesenteric lymph nodes after the resection of biliary malignancies (19). Therefore, the coincidence of biliary obstruction and BT may be a common clinical situation in patients with hepatobiliary pancreatic malignancies.

Several experimental studies have demonstrated severe liver damage under conditions of biliary obstruction (induced by BDL) with endotoxin (LPS) challenge (2, 4, 17, 18). However, those studies administered LPS through the intravenous (2, 15), intraintestinal (18), intrabiliary (4), or intraperitoneal (17) routes. To accurately reproduce clinical conditions of biliary obstruction and BT, we developed a rat model of BDL with LPS injection via the portal vein. In this model, considerable liver damage was observed 2–4 h after LPS injection. Even with a very small amount of injected LPS (0.5 µg) compared with other studies (2, 4, 15, 17, 18), one-half of the rats died within 24 h. The serum AST, ALT, and proinflammatory cytokine levels were markedly elevated 2–4 h after LPS injection. These results indicated that, after biliary obstruction, the liver is highly susceptible to LPS inflow via the portal circulation (i.e., BT), and very strong inflammatory responses and liver damage are provoked under such conditions.

Biliary drainage is a common procedure in patients with biliary obstruction (8, 10). There are many clinical studies that have discussed the risks and benefits of biliary drainage for...
patients with obstructive jaundice (9, 23, 29, 30). In the meta-analyses, there were no significant differences in mortality in the preoperative biliary drainage group vs. the direct surgery group (30). The overall serious morbidity was even higher in the preoperative biliary drainage group, most likely because of procedure-related complications and cholangitis (7, 29). However, in cases of perihilar cholangiocarcinoma, in which the patients must undergo a major hepatectomy with extrahepatic bile duct resection, whether preoperative biliary drainage is necessary is still controversial (20). According to the results in this study, the performance of preoperative biliary drainage in patients with obstructive jaundice is strongly recommended because these patients are highly susceptible to sepsis and may develop severe liver damage after highly invasive surgery, which induces BT.

LPS binds to TLR4 and transduces subsequent signals that lead to upregulation of the inflammatory response (2, 18). As was observed in previous studies, we demonstrated an upregulation of TLR4 in the liver after 1 wk of BDL (4, 16, 18). Immunostaining revealed strong TLR4 expression along the sinusoid in the BDL group (Fig. 2D). To further identify the cells that specifically express TLR4, we performed double immunostaining. The expression of TLR4 was mostly colocalized in Kupffer cells (Fig. 4). More interestingly, the number of Kupffer cells was significantly increased in BDL rats. These changes were normalized or attenuated only one day after biliary decompression with the biliary drainage procedure. To the best of our knowledge, this is the first report that has demonstrated a dynamic change in TLR4 expression and the number of Kupffer cells in the liver depending on biliary obstruction. Interestingly, dynamic changes of endotoxin levels in the serum and liver after intraportal administration of LPS were similar (Fig. 5, A and C). In contrast, there was almost no detection of endotoxin in the urine and lung (Fig. 5, B and D). These results implied that the LPS administered through the portal vein were mainly captured in the liver. Nevertheless, the levels of endotoxin in the serum and liver tended to be lower in the BDL group than the Sham group. The exact mechanism of LPS metabolism after intraportal administration should be elucidated in a future study.

It was also clearly demonstrated that blocking of TLR4 signaling (by TAK-242) or Kupffer cell activation (by GdCl3) effectively reduced liver damage after the second attack of BT in biliary obstruction to a level that was similar to that after biliary drainage. GdCl3, a Kupffer cell deactivator, is not clinically available. However, the TAK-242 used in this study has been extensively used in humans and may be a promising therapeutic drug because it strongly attenuated LPS-induced liver damage. Obviously, biliary drainage is the most appropriate way to relieve hypersensitivity to endotoxin in BDL rats. However, in some clinical settings, biliary drainage is difficult to perform. Biliary drainage itself may also be accompanied by procedure-related side effects (29) and biliary tract infection (14, 27). In such cases, we recommend the use of TAK-242 to inhibit detrimental liver damage.

![Sham BDL Drainage](http://apjgi.physiology.org/doi/10.1152/apjgi.00366.2013/fig6)

Fig. 6. Histology of the livers in the Sham, BDL, and Drainage groups. The micrographs depict representative hematoxylin and eosin staining of paraffin-embedded liver slides, recorded using a ×4 objective. The percentage of liver necrosis in five low-powered (×10 objective) fields of view for each rat was counted. *P < 0.05 vs. Sham, †P < 0.05 vs. BDL. N = 6 in each group.
It should be noted, however, that a phase III clinical study using TAK-242 for patients with severe sepsis-induced shock or respiratory failure failed to suppress inflammatory cytokine levels (22). It was concluded that TAK-242 was not useful in patients who developed severe sepsis. However, in this study, TAK-242 was administered after the patients developed severe sepsis. Moreover, the cause of sepsis was too variable, including lung, urinary tract, and intra-abdominal infections. It may be overly hasty to make a definite conclusion for the usefulness of TAK-242 from only one clinical study. Based on our findings, we propose the use of TAK-242 as a preconditioning drug for patients with biliary obstruction who must undergo highly invasive surgery that frequently induces BT. In fact, in other animal models, pretreatment with TAK-242 significantly improved the survival rate in a porcine peritonitis model (5) and a murine endotoxemia model (25).

In conclusion, our study demonstrated an increased expression of TLR4 protein as well as increased numbers of Kupffer cells in the liver of BDL rats, and these changes were effectively normalized after biliary drainage. The expression of TLR4 was mainly colocalized with Kupffer cells. A marked increase in serum liver enzyme and proinflammatory cytokine levels was observed after intraportal LPS administration in BDL rats. However, these changes were significantly attenuated by pretreatment with TAK-242 to a level that was similar to those seen with biliary drainage. We propose the use of TAK-242 as a novel therapeutic drug for patients with biliary obstruction who are susceptible to BT.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

REFERENCES


