LPS signaling enhances hepatic fibrogenesis caused by experimental cholestasis in mice

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Isayama, Fuyumi, Ian N. Hines, Michael Kremer, Richard J. Milton, Christy L. Byrd, Ashley W. Perry, Stephen E. McKim, Christopher Parsons, Richard A. Rippe, and Michael D. Wheeler. LPS signaling enhances hepatic fibrogenesis caused by experimental cholestasis in mice. Am J Physiol Gastrointest Liver Physiol 290: G1318 –G1328, 2006. First published January 26, 2006; doi:10.1152/ajpgi.00405.2005.—Although it is clear that bile acid accumulation is the major initiator of fibrosis caused by cholestatic liver disease, endotoxemia is a common side effect. However, the depletion of hepatic macrophages with gadolinium chloride blunts hepatic fibrosis. Because endotoxin is a key activator of hepatic macrophages, this study was designed to test the hypothesis that LPS signaling through CD14 contributes to hepatic fibrosis caused by experimental cholestasis. Wild-type mice and CD14 knockout mice (CD14−/−) underwent sham operation or bile duct ligation and were killed 3 wk later. Measures of liver injury, such as focal necrosis, biliary cell proliferation, and inflammatory cell influx, were not significantly different among the strains 3 wk after bile duct ligation. Markers of liver fibrosis such as Sirius red staining, liver hydroxyproline, and α-smooth muscle actin expression were blunted in CD14−/− mice compared with wild-type mice after bile duct ligation. Despite no difference in lymphocyte infiltration, the macrophage/monocyte activation marker OX42 (CD11b) and the oxidative stress/lipid peroxidation marker 4-hydroxynonenal were significantly upregulated in wild-type mice after bile duct ligation but not in CD14−/− mice. Increased profibrogenic cytokine mRNA expression in the liver after bile duct ligation was significantly blunted in CD14−/− mice compared with the wild type. The hypothesis that LPS was involved in experimental cholestatic liver fibrosis was tested using mice deficient in LPS-binding protein (LBP−/−). LBP−/− mice had less liver injury and fibrosis (Sirius red staining and hydroxyproline content) compared with wild-type mice after bile duct ligation. In conclusion, these data demonstrate that endotoxin in a CD14-dependent manner exacerbates hepatic fibrogenesis and macrophage activation to produce oxidants and cytokines after bile duct ligation.

LPS signaling causes the activation of macrophages and their release of cytokines and chemical oxidants, such as superoxide 

Although the mechanisms of cholestasis-induced liver fibrosis are unclear, obstruction of bile flow causes the accumulation and retention of hydrophobic bile salts in the liver, which are toxicants to a number of cells, including hepatocytes and ductal biliary epithelial cells (1, 20). It has been hypothesized that Fas-mediated apoptosis of hepatocytes facilitates hepatic stellate cell activation and subsequent hepatic fibrogenesis (4). Biliary cell proliferation associated with cholestatic liver disease occurs seemingly as the direct effects of bile acids. On the other hand, cytokines and growth factors are also important in the activation of hepatic stellate cells. Canbay and colleagues (3) have also reported that activated Kupffer cells resulting from apoptotic bodies contribute to the fibrogenic stimulus. Depletion of Kupffer cells with gadolinium chloride prevents bile duct ligation-induced liver injury, hepatic stellate cell activation, and neutrophil infiltration. A recent report suggested that Kupffer cells demonstrate significant increase in phagocytic activity, oxidative burst potential, and cytokine production after bile duct ligation (14). Moreover, it was found after bile duct ligation that Kupffer cells were more sensitive to the effects of lipopolysaccharide (LPS)-binding protein (LBP), a soluble protein that increases the affinity and potency of LPS to macrophages.

In other models of hepatic fibrosis, such as carbon tetrachloride-induced and dimethylnitrosamine (DMN)-induced rat liver fibrosis, depletion of Kupffer cells with gadolinium chloride prevented liver fibrosis (15, 17, 19). It was hypothesized that cytokines, oxidants, and growth factors secreted by activated Kupffer cells stimulate quiescent hepatic stellate cells to produce collagen. Specifically, transforming growth factor (TGFB)-β and tumor necrosis factor (TNF)-α from both hepatic macrophages and other liver cells, including stellate cells, are critically involved in fibrogenesis in experimental cholestatic liver disease and other models of hepatic fibrosis (2).

It has been demonstrated that endotoxemia often occurs together with cholestasis (5, 8), suggesting a potential relationship between bile salt and endotoxemia in cholestasis. Indeed, it has been reported that obstructive jaundice can increase bacterial translocation (9). LPS is clearly capable of enhancing liver disease by activating Kupffer cells and exacerbating cytokine production, but the precise contribution of LPS to hepatic fibrosis is not known. Thus it was hypothesized that LPS activation of hepatic macrophages in experimental cholestatic liver disease.

Because CD14 is essential for LPS signaling, CD14-deficient mice were used to test the hypothesis that LPS is involved in liver fibrosis caused by bile duct ligation. Moreover, LPS signaling through CD14 and Toll-like receptor 4 (TLR4) is facilitated by soluble LBP, which increases the affinity between LPS and its receptor. Thus mice deficient in LBP (LBP−/−) were also used to test the hypothesis that LPS signaling through CD14 contributes to hepatic fibrogenesis.
C57BL/6-Lbp/-mlBuru) mice (25–30 g) were obtained from The Jackson Laboratory.

**MATERIALS AND METHODS**

**Signaling contributed to fibrogenesis caused by bile duct ligation.**

**MATERIALS AND METHODS**

**Animals.** Male wild-type (WT; C57BL/6J), CD14 knockout (CD14−/−; B6.129S-Cd14tm1Flm1), and LBP knockout (LBP−/−; C57BL/6-Lbp−/−Buru) mice (25–30 g) were obtained from The Jackson Laboratory (Bar Harbor, ME). All animals were housed in pathogen-free barrier facilities accredited by the American Association for Accreditation of Laboratory Animal Care, and procedures used were approved by the university Institutional Animal Care and Use Committee.

**Surgery.** WT, CD14−/−, and LBP−/− mice were randomly divided into two experimental groups that underwent sham operation or bile duct ligation. Operations were performed as described elsewhere (10). Briefly, under pentobarbital sodium anesthesia (75 mg/kg), the peritoneal cavity was opened, and the common bile duct was triple ligated with 7-0 silk suture and cut between the second and third ligatures close to the liver. Sham operations were performed by gently touching the bile duct three times. Animals were killed 3 wk after surgery under pentobarbital sodium anesthesia (Nembutal, 60 mg/kg ip).

**Liver hydroxyproline content.** The hepatic hydroxyproline content was measured using the method described elsewhere (12). Briefly, liver samples (80–150 mg) were dried up, powdered in liquid nitrogen, and hydrolyzed. The sample was mixed with one-half volume of chloramine T solution (1.41 g sodium p-chloro-phenol, and 30 ml of 60% perchloric acid) was added slowly. Samples were incubated at 60°C for 15 min and read at 561 nm in a spectrophotometer.

**Pathological evaluation.** Liver sections were stained by hematoxylin and eosin for the detection of inflammation or necrosis. For histological analysis, sections were evaluated by an independent, blinded reviewer. Sections were scored for level of necrosis (0, no observable necrosis; 1, random cellular necrosis; 2, necrotic area 10–25%; 3, necrotic area 25–50%; 4, necrotic area >50%) and numbers of lymphocyte foci from 10 randomly selected low-power fields.

**RESULTS**

**Table 1. Routine parameters after sham operation or BDL**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CD14−/−</th>
<th>LBP−/−</th>
</tr>
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<tbody>
<tr>
<td>Body weight gain, g/3 wk</td>
<td>2.1 ±0.4</td>
<td>2.1 ±0.2</td>
<td>2.1 ±0.2</td>
</tr>
<tr>
<td>Liver-to-body weight ratio, %</td>
<td>4.5 ±0.2</td>
<td>4.7 ±0.3</td>
<td>4.7 ±0.3</td>
</tr>
<tr>
<td>Total bilirubin, IU/l</td>
<td>0.6 ±0.1</td>
<td>0.7 ±0.3</td>
<td>0.6 ±0.1</td>
</tr>
<tr>
<td>Mortality, ratio</td>
<td>0/10</td>
<td>0/8</td>
<td>0/8</td>
</tr>
<tr>
<td>ALP, IU/l</td>
<td>114 ±16</td>
<td>ND</td>
<td>77 ±9</td>
</tr>
<tr>
<td>ALT, IU/l</td>
<td>24 ±4</td>
<td>23.4</td>
<td>32 ±4</td>
</tr>
<tr>
<td>Necrosis (0–4)</td>
<td>0</td>
<td>0.17 ±0.1</td>
<td>0</td>
</tr>
<tr>
<td>Inflammation, loci/field</td>
<td>0.2 ±0.1</td>
<td>0.17 ±0.1</td>
<td>0</td>
</tr>
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</table>

Data represent means ± SE; n = 8–12 mice in each group. BDL, bile duct ligation; WT, wild type; LBP, lipopolysaccharide-binding protein; ALP, alkaline phosphatase; ALT, alanine transaminase. ND, not determined. *P < 0.05 compared with mice with sham operation and †P < 0.05 compared with WT mice that underwent BDL (by two-way ANOVA using Bonferroni’s post hoc test).

**Fig. 1.** CD14-deficient (CD14−/−) mice have blunted pathology 3 wk after bile duct ligation (BDL). Representative pathology (original magnification ×200) of wild-type (WT) and CD14−/− mice 3 wk after sham operation or BDL is shown. Liver sections were collected and stained using hematoxylin and eosin (H&E). A: WT mice after sham operation; B: CD14−/− mice after sham operation; C: WT mice after BDL; D: CD14−/− mice after BDL.
fields/section. Sections were stained with saturated picric acid containing 0.1% Sirius red and 0.1% fast green. A Universal Imaging (Metamorph) image acquisition and analysis system was used to capture and analyze the stained tissue sections at ×200 magnification. Data from each tissue section (randomly selected 10 fields/section) were pooled to determine means.

**Immunohistochemistry.** For immunohistochemical staining, formalin-fixed tumor sections were deparaffinized and rehydrated by standard protocols and were incubated with specific primary antibodies. For CD11b (Serotec, Raleigh, NC), antibody at a dilution of 1:50 in 1% BSA was applied to sections for 2 h at 37°C. For 4-hydroxynonenal (4-HNE; Alpha Diagnostics, San Antonio, TX), antibody at a dilution of 1:250 was used. For CD3 and intercellular adhesion molecule (ICAM)-1 (Santa Cruz Biotechnologies, Santa Cruz, CA), antibodies were diluted 1:200 in 1% BSA and incubated on sections overnight at 4°C. For myeloperoxidase immunohistochemistry, antibody was diluted 1:200 in 1% BSA and applied to sections for 2 h before secondary staining. Staining was visualized using the horseradish peroxidase-conjugated DAKO staining system (DAKO InVision, Carpenteria, CA).

**Western blot assay.** Whole liver extracts (40 μg) were separated by 8–16% SDS gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk-Tween 20-Tris-buffered saline for 1 h. Membranes were then immunoblotted with antibodies against α-smooth muscle actin (α-SMA; DAKO) diluted 1:1,000 in 1% blocking solution for 1 h or with antibodies against ICAM-1 (Santa Cruz Biotechnologies) diluted 1:1,000 in 1% blocking solution for 1 h. Secondary antibodies conjugated to horseradish peroxidase (1:5,000 in 1% blocking solution) were used, and chemiluminescence was used to visualize immunoblots.

**RNase protection assay.** Total RNA was isolated from liver tissue using TRIzol (Invitrogen). RNase protection assays were performed using the RiboQuant multiprobe assay system (Pharmingen). Briefly, [32P]RNA probes were transcribed with T7 polymerase using the multiprobe template set mCK-3b. RNA (20 μg) was hybridized with 4 × 10⁶ counts/min of probe overnight at 56°C. Samples were then digested with RNase followed by proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Samples were resolved on a 5% acrylamide-bisacrylamide (19:1) urea gel. After drying, the gel was visualized by autoradiography.

Fig. 2. Liver Sirius red staining is blunted in CD14−/− mice after BDL. A: representative photomicrographs of Sirius red staining (original magnification ×10). WT and CD14−/− mice 3 wk after sham operation or BDL. Liver sections were collected and stained as described in MATERIALS AND METHODS. A:A: WT mice after sham operation; A:B: CD14−/− mice after sham operation; A:C: WT mice after BDL; A:D: CD14−/− mice after BDL. B: percentage of area stained was measured by image analysis from 10 random fields from each tissue section, and data were pooled to determine means. *P < 0.05 compared with mice after sham operation and **P < 0.05 compared with WT mice after BDL.
IN Volvement of CD14 in Hepatic fibrosis

Changes in routine parameters after bile duct ligation.

Routine parameters after either sham operation or bile duct ligation are summarized in Table 1. In mice that underwent sham operation, serum alanine transaminase (ALT) levels were ~30 U/l in each strain. In WT mice, serum ALT levels were significantly increased to ~200 U/l after bile duct ligation. This increase was significantly blunted by >40% in CD14−/− mice, supporting the hypothesis that LPS may contribute to liver injury under conditions known to induce hepatic fibrosis. To determine the degree of necrosis after bile duct ligation, histology was evaluated in liver sections from WT and CD14−/− mice (Fig. 1). It was hypothesized that the deletion of CD14 would blunt bile duct ligation-induced pathogenesis. There were no pathological changes observed in sham groups. However, patchy necrosis was observed in WT mice after bile duct ligation. Moreover, increased numbers of inflammatory cells were seen around the portal areas of WT mice after bile duct ligation. There was an apparent liver architectural change, consistent with biliary cell proliferation and the formation of bridging fibrosis in WT livers as well. Histology was evaluated and scored on the basis of inflammatory cell influx (0–4) and the extent of necrosis (0–4). The level of necrotic tissue was significantly increased in WT mice after bile duct ligation. The necrosis was slightly but significantly blunted in CD14−/− mice. Interestingly, it was demonstrated that inflammatory cell foci resulting from bile duct ligation were not different among the strains (Table 1). Because experimental cholestasis induces biliary cell proliferation, cell proliferation was assessed by staining liver sections with antibodies against proliferating cell nuclear antigen. It was noted that bile duct cell proliferation was markedly increased after bile duct ligation, but the extent of cell proliferation was not different among the strains (data not shown). Importantly, with the exception of detectable biliary cell proliferation, these pathological changes were attenuated in CD14−/− mice after bile duct ligation (Fig. 1, C and D). These data strongly support the hypothesis that LPS signaling is critically involved in pathology (i.e., patchy necrosis) associated with experimental cholestasis and suggest that LPS may play a contributory role in fibrogenesis as well.

Effects of bile duct ligation on liver fibrogenesis. Sirius red staining in liver sections from WT and CD14−/− mice 3 wk after sham operation or bile duct ligation was evaluated (Fig. 2A). Image densitometry was performed to quantify the changes in liver fibrosis resulting from bile duct ligation (Fig. 2B). Mild positive staining (red) was observed around the portal regions in both strains. However, positive staining was increased significantly in WT mice with bile duct ligation. Positive staining was decreased significantly in CD14−/− mice after bile duct ligation compared with WT mice. This positive staining was reduced especially in the area of midzonal and pericentral vein in CD14−/− mice after bile duct ligation. These data, combined with the changes in hydroxyproline content, support the hypothesis that LPS through CD14 exacerbates hepatic fibrosis caused by cholestatic liver disease in mice.

Fig. 3. Liver hydroxyproline is blunted after BDL in CD14−/− mice. WT and CD14−/− mice underwent sham operation or BDL for 3 wk. Hydroxyproline content from dried liver tissue was determined as described in MATERIALS AND METHODS. Data are representative of 4–8 tissues/group measured as replicates and are expressed as means ± SE. *P < 0.05 compared with mice after sham operation.

Statistics. Two-way ANOVA with Bonferroni’s post hoc test was used for the determination of statistical significance. Data are presented as means ± SE. P < 0.05 was considered as significant.

RESULTS

Changes in routine parameters after bile duct ligation.

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The hepatic content of hydroxyproline correlates with the degree of liver fibrosis (12). Therefore, hydroxyproline was measured to test the hypothesis that the deletion of CD14 would blunt bile duct ligation-induced hepatic fibrosis. Hydroxyproline content was \( \sim 0.3 \mu g/mg \) in dried liver tissue after sham operation; these levels were increased significantly by >10-fold 3 wk after bile duct ligation (Fig. 3). Hydroxyproline levels were blunted in CD14\(^{-/-}\) mice compared with WT mice after bile duct ligation. Although not statistically different, the increase in hydroxyproline was blunted in CD14\(^{-/-}\) mice compared with WT mice.

As a direct marker of hepatic stellate cell activation, liver extracts from WT and CD14\(^{-/-}\) mice after sham or bile duct ligation were evaluated for \( \alpha\)-SMA expression by Western blot analysis (Fig. 4). Bile duct ligation in WT mice led to a marked upregulation of \( \alpha\)-SMA compared with sham controls. However, in CD14\(^{-/-}\) mice, the increase in \( \alpha\)-SMA after bile duct ligation was less pronounced. These data suggest that LPS signaling through CD14 is involved in the activation of hepatic stellate cells.

**Effect of bile duct ligation on lymphocyte infiltration.** Experimental cholesitatic liver fibrosis is also associated with lymphocyte infiltration, likely as a direct result of hepatocellular and biliary cell injury. Because the LPS response through CD14 is not exclusively, but largely, a macrophage response, it was not expected that deletion of CD14 would have any effect on lymphocyte infiltration under these conditions. To assess the extent of lymphocyte recruitment, liver sections were...
stained immunohistochemically with anti-CD3 antibody (Fig. 5A). Livers from WT and CD14−/− mice under normal conditions expressed very little CD3 as expected. However, 3 wk after bile duct ligation, the level of CD3+ cells was increased dramatically. The increase in CD3+ cells was observed in both WT and CD14−/− mice. As an indicator of neutrophil infiltration, liver sections were stained with antibodies against myeloperoxidase (Fig. 5B). No differences in myeloperoxidase expression in liver were observed after bile duct ligation between either WT or CD14-deficient animals.

Importantly, the recruitment of inflammatory cells involves the expression of key adhesion molecules such as ICAM-1. Thus the expression of ICAM-1 was determined by Western blot analysis (Fig. 5C). In livers from WT and CD14−/− mice, no expression of ICAM-1 was observed under normal conditions. However, after bile duct ligation, the expression of ICAM-1 was easily detectable by Western blot analysis in liver extracts from both WT and CD14−/− mice. There were no differences, however, between the two strains, a finding consistent with the fact that lymphocyte and neutrophil infiltration was also not different between WT and CD14−/− mice.

Taken together, these data suggest that the reduction in cholestasis-induced liver fibrosis in CD14−/− mice is not necessarily because they have impaired recruitment of inflammatory cells. Interestingly, it is likely that the upregulation of adhesion molecule ICAM and lymphocyte infiltration is because of the direct effect of hepatocellular and sinusoidal endothelial cell damage. Moreover, it is possible that the CD14-independent process is responsible for the upregulation of adhesion molecules, for example, because of bile acid toxicity or TLR2 activation by bacterial translocation.

Macrophage activation marker CD11b immunohistochemistry. It is hypothesized that LPS activates hepatic macrophages to elicit a profibrogenic response. To determine whether LPS

Fig. 6. Expression of CD11b is reduced in CD14−/− mice after BDL. A: representative photomicrographs of CD11b (CR3) immunohistochemical staining (original magnification ×40) in liver sections from WT (A,A and A,C) and CD14−/− mice (A,B and A,D) 3 wk after sham operation (A,A and A,B) or BDL (A,C and A,D). Liver sections were collected and stained as described in MATERIALS AND METHODS. B: quantification of CD11b staining using Image J software from 5 randomly selected ×40 fields/slide with 3 sections/group. *P < 0.05 compared with mice after sham operation and †P < 0.05 compared with WT mice after BDL.
activates hepatic macrophages after bile duct ligation, liver sections were stained using antibodies against CD11b, because it has been shown that LPS rapidly upregulates the expression of CD11b almost exclusively on macrophages in the liver (11). In WT mice, CD11b was markedly increased in liver 3 wk after bile duct ligation compared with sham operation (Fig. 6A). Moreover, staining was primarily localized in nonparechymal cells, presumably hepatic macrophages. Image analysis revealed a significant fivefold increase in the level of CD11b staining in WT mice after bile duct ligation (Fig. 6B). The increase in CD11b staining was nearly completely blocked in CD14−/− mice 3 wk after bile duct ligation compared with WT mice. These data support the hypothesis that hepatic macrophages are indeed activated after bile duct ligation and that cell activation is dependent on LPS signaling. These data also suggest that LPS signaling may contribute to fibrogenesis through activating macrophages.

Effect of bile duct ligation on hepatic oxidative damage. Several groups have demonstrated that hepatic fibrosis is associated with increases in oxidant production (21). It is hypothesized here that LPS contributes to oxidative stress caused by bile duct ligation in mice. Liver sections from WT mice and CD14−/− mice were immunohistochemically stained using antibodies against 4-HNE, a predominant lipid peroxidation product of oxidative stress. A measureable increase in 4-HNE staining was observed in WT mice livers 3 wk after bile duct ligation (Fig. 7) compared with sham operation. This increase resulting from bile duct ligation was blunted in CD14−/− mice, suggesting that LPS signaling may contribute to oxidative stress during experimental cholestasis.

Effect of bile duct ligation on profibrogenic cytokine expression. Predominant profibrogenic cytokines TNF-α and TGF-β that are produced in response to LPS may play an important role in the activation of hepatic stellate cells. Because Kupffer cells are the primary source of cytokines in an early hepatic innate immune response and because hepatic macrophages are activated after bile duct ligation, it was hypothesized that profibrogenic cytokine expression would be blunted in CD14−/− mice after bile duct ligation. Cytokine expression from WT and CD14−/− mice was evaluated by RNase protection assay 4 and 21 days after sham operation or bile duct ligation. At 21 days, the changes in cytokine expression were observed but insignificant. This is likely because of the fact that the proinflammatory events occur early in the mechanisms of fibrogenesis. For this reason, cytokines were also measured at 4 days after bile duct ligation. Cytokine mRNA expression was barely detectable in both groups after sham operation (Fig. 8). However, the expression of TNF-α, interferon-γ, and, most importantly, TGF-β mRNA was increased significantly in WT mice after bile duct ligation. Consistent with this hypothesis, the increase in TNF-α production was blunted significantly in CD14−/− mice by >50% after bile duct ligation compared with WT mice.

Loss of LBP protects against experimental cholestatic liver injury. To further examine the direct role of endotoxin signaling in experimental cholestatic liver disease, LBP−/− were used. LBP is a serum protein that carries free endotoxin in blood and increases the affinity of LPS to its receptor complex CD14:TLR4. WT and LBP−/− mice underwent either sham operation or bile duct ligation. Serum transaminase levels were increased significantly after 3 wk in WT mice after bile duct ligation compared with control mice (Table 1). The increase in ALT was blunted significantly in LBP−/− mice after bile duct ligation compared with WT mice. Liver pathology was evaluated 3 wk after bile duct ligation (Fig. 9). Livers from WT mice, as expected, were severely necrotic and showed signif-

Fig. 7. CD14−/− mice have blunted lipid peroxidation product 4-hydroxynonenal (4-HNE) after BDL. A: representative photomicrographs of 4-HNE immunohistochemical staining (original magnification ×20) in liver sections from WT (A and C) and CD14−/− mice (B and D) 3 wk after sham operation (A and B) or BDL (C and D). Liver sections were collected and stained as described in MATERIALS AND METHODS.
The liver pathology observed in LBP<sup>−/−</sup> mice after bile duct ligation closely resembles that seen in CD14<sup>−/−</sup> mice. These data corroborate the notion that endotoxin through CD14 plays an important role in liver injury associated with cholestasis.

**DISCUSSION**

Cholestatic liver disease ultimately leads to fibrosis primarily because of obstruction of bile flow and accumulation of hydrophobic bile salts in liver that are capable of causing cellular damage to hepatocytes and ductal biliary epithelial cells (1, 6, 13). Several studies have reported that biliary cell injury is characterized by the coexistence of cell loss by apoptotic or lytic cell death with biliary cell proliferation and various degrees of portal inflammation. Bile acids also induce hepatocyte apoptosis, and it has been proposed that Fas-mediated apoptosis of hepatocytes facilitates hepatic stellate cell activation (4). These data suggest that pathological factors associated with cholestatic liver disease (i.e., biliary cell proliferation, inflammatory cell recruitment, and some necrosis) occur as a direct effect of bile acids.

It has also been reported that cytokines and growth factors are necessary for the activation of hepatic stellate cells in fibrogenesis. Canbay and colleagues (3) have also suggested that Kupffer cells are activated as a result of engulfing apoptotic bodies and are partially responsible for the fibrogenic stimulus. Depletion of macrophages with gadolinium chloride prevented bile duct ligation-induced liver injury and hepatic stellate cell activation.

In other models of hepatic fibrosis, it has also been shown that depletion of Kupffer cells with gadolinium chloride prevented bile duct ligation-induced liver injury and hepatic stellate cell activation. Because CD14<sup>−/−</sup> mice had a blunt fibrogenic response from bile duct ligation, it was hypothesized that LBP<sup>−/−</sup> mice would be similarly protected against bile duct ligation-induced liver fibrosis. As above, livers from WT and LBP<sup>−/−</sup> mice that underwent sham or bile duct ligation were stained with Sirius red to determine the extent of liver fibrosis (Fig. 10A). Livers from WT mice had a significant amount of Sirius red staining, indicating collagen production. The Sirius red staining was localized predominantly in periportal and midzonal regions. Although staining in the livers of LBP<sup>−/−</sup> mice was observed, the extent of staining was reduced significantly (Fig. 10B).

The extent of liver fibrosis was reflected in the amount of hydroxyproline content. An increase in hydroxyproline in livers of WT mice after bile duct ligation was observed. Moreover, the increase was blunted significantly in livers of LBP<sup>−/−</sup> animals (Fig. 10C). The changes in hydroxyproline correlated strongly with the extent of Sirius red staining in livers. These data strongly support the hypothesis that LPS contributes to the fibrogenic response caused by experimental cholestatic liver disease.

**References**

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**Figure Legends**

**Fig. 8. Cytokine expression in liver is reduced in CD14<sup>−/−</sup> mice after BDL.**

A: Liver mRNA from either WT or CD14<sup>−/−</sup> mice after either sham operation or BDL was evaluated by RNase protection assay using the mCK-3b multiprobe template (BD Pharmingen) as described in MATERIALS AND METHODS. Undigested probe (lane 1) and protected probe fragments were separated on a 6% bis-acrylamide-acrylamide (29:1) gel and visualized by phosphoimaging. B: Image densitometry for the relative intensity of each band was determined using Image J software. The relative changes in tumor necrosis factor (TNF)-α, interferon (IFN)-γ, transforming growth factor (TGF)-β, and macrophage-inducing factor (MIF) compared with the expression of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown. LTB, Data are expressed as means ± SE of the degree of change relative to GAPDH expression. *P < 0.05 compared with mice after sham operation and **P < 0.05 compared with WT mice after BDL.
The contribution of LPS to hepatic fibrosis is not known. Thus it was hypothesized that LPS activated hepatic macrophages in experimental cholestatic liver disease. In this report, hepatic macrophages are indeed activated after bile duct ligation (Fig. 6). The expression of CD11b, a component of the C3 complement receptor primarily expressed on myeloid cells (i.e., macrophages and monocytes), used to determine the extent of macrophage activation, was enhanced significantly in mice after bile duct ligation. These data support earlier findings by Canbay and colleagues (3). The point to this work is not to demonstrate hepatic activation after bile duct ligation but to investigate whether endotoxin plays a role in macrophage activation under these conditions and whether LPS signaling contributes to the fibrotic response. However, it was demonstrated here that the increase in CD11b expression after bile duct ligation was nearly completely blunted in animals lacking CD14. These data support the hypothesis that LPS indeed plays a role in the activation of macrophages under these conditions.

An interesting finding is that no strong differences in inflammation and biliary cell proliferation were noted among the strains used in this study, suggesting that these effects of biliary obstruction occur independent of CD14-dependent mechanisms. A possible explanation could be the direct toxicity of bile acid accumulation, which is known to induce hepatocellular injury and biliary cell proliferation. Moreover, the increase in adhesion molecules could be the result of CD14-TLR4-independent but TLR2-dependent mediated events. Because endotoxemia and bacterial translation are likely increased in cholestasis, it is likely that these products activate other inflammatory signaling pathways involving TLR2 and other immune receptors on a variety of cell types in liver. However, there is a debate whether neutrophil infiltration is a necessary component of fibrosis caused by bile duct ligation. Gugral group (7) showed that ICAM−/− mice showed a slight improvement in liver injury after bile duct ligation (7), where it was recently reported that depletion of neutrophils had no significant effect on bile duct ligation-induced fibrosis (18).

The cytokine response after bile duct ligation involved the upregulation of TNF-α, interferon-γ, and macrophage-inducing factor. Interestingly, the upregulation of these cytokines and chemokines was blunted significantly in CD14-deficient mice after bile duct ligation (Fig. 8). Of most importance to the fibrosis was the observation that TGF-β, a strong fibrogenic cytokine, was nearly completely inhibited. The production of these factors are not exclusively macrophage derived but, at least, suggest that macrophages are involved. Clearly, the fact that their production is blunted in CD14-deficient mice suggests that cytokine production after bile duct ligation is caused by LPS. Whether cells are activated by bile acids or through the process of engulfing apoptotic bodies as suggested above or by other mechanisms is possible but not clear.

It is apparent that cholestasis is sufficient to induce hepatocellular injury and inflammation, resulting in biliary cell proliferation and fibrosis. However, studies have reported that depletion of macrophages with GdGl3 prevented hepatic fibrosis in a number of models (3). Thus the idea that eliminating LPS signaling through CD14 would alter the fibrotic response caused by bile duct ligation was addressed. Reduction of tissue hydroxyproline, collagen deposition, and α-SMA expression in CD14 mice clearly supports this notion. The conclusion is that the LPS-induced cytokine response from macrophages enhances fibrogenesis caused by bile acid retention in this model. An argument against this hypothesis is the assumption that
simply decreasing inflammation by blocking endotoxin signaling would attenuate fibrosis. However, it is demonstrated here that, despite inflammation, fibrosis was still blunted in mice lacking the endotoxin receptor. These data suggest that the role of endotoxin is not associated with or dependent on inflammation and suggest that it is related to the hepatic macrophage response. An alternative consideration is based on the observation made by Paik and colleagues (16) that endotoxin could directly activate hepatic stellate cells to produce collagen. Based on previously published GdCl₃ experiments, one would expect that if LPS directly activated stellate cells to a significant level in vivo, then depletion of Kupffer cells would not protect against fibrosis. Taken together, these data suggest that, although LPS may activate stellate cells to some degree, LPS stimulates hepatic cytokine production predominantly through macrophages.

Because CD₁₄−/− mice were less susceptible to hepatic fibrosis, it was concluded that LPS plays a contributory role in fibrogenesis. Bile acid retention alone is likely sufficient to induce hepatocellular injury, bile duct proliferation, a mild inflammatory response, and some fibrosis. However, it is hypothesized that LPS, which signals through the CD₁₄ receptor, activates macrophages and initiates oxidant production and the cytokine response. Data presented here clearly demonstrate that LPS plays a role in macrophage activation and the production of cytokines after bile duct ligation in mice. More importantly, these data strongly support the hypothesis that LPS signaling contributes to hepatic fibrosis in mice.

GRANTS
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REFERENCES


