TLR4 enhances TGF-β signaling and hepatic fibrosis

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Hepatic injury is associated with a defective intestinal barrier and increased hepatic exposure to bacterial products. Here we report that the intestinal bacterial microflora and a functional Toll-like receptor 4 (TLR4), but not TLR2, are required for hepatic fibrogenesis. Using Tlr4-chimeric mice and in vivo lipopolysaccharide (LPS) challenge, we demonstrate that quiescent hepatic stellate cells (HSCs), the main precursors for myofibroblasts in the liver, are the predominant target through which TLR4 ligands promote fibrogenesis. In quiescent HSCs, TLR4 activation not only upregulates chemokine secretion and induces chemotaxis of Kupffer cells, but also downregulates the transforming growth factor (TGF)-β pseudoreceptor Bambi to sensitize HSCs to TGF-β-induced signals and allow for unrestricted activation by Kupffer cells. LPS-induced Bambi downregulation and sensitization to TGF-β is mediated by a MyD88–NF-κB–dependent pathway. Accordingly, Myd88-deficient mice have decreased hepatic fibrosis. Thus, modulation of TGF-β signaling by a TLR4-MyD88–NF-κB axis provides a novel link between proinflammatory and profibrogenic signals.

Chronic tissue injury leads to fibrosis in many organs, including the liver, lung, kidney and heart. In chronic liver disease, development of fibrosis is the first step toward the progression to cirrhosis and its complications (such as organ failure, esophageal variceal bleeding and hepatocellular carcinoma), independently of the underlying etiology. TGF-β and platelet-derived growth factor (PDGF) have been characterized as key cytokines that mediate hepatic fibrogenesis, but the current model of TGF-β– and PDGF-dependent fibrogenesis does not account for the role of inflammatory mediators in hepatic fibrogenesis. Kupffer cells, the resident hepatic macrophages, and hepatic stellate cells (HSCs), the main producers of extracellular matrix in the fibrotic liver, are key in hepatic fibrogenesis and are both targets of proinflammatory mediators. Although inflammation is present in virtually all patients with hepatic fibrosis and correlates with fibrosis progression, the molecular link between hepatic inflammation and fibrogenesis remains elusive.

TLRs comprise a highly conserved family of receptors that recognize pathogen-associated molecular patterns and allow the host to detect microbial infection. TLRs are not only important in the regulation of innate and adaptive immune responses, but also involved in noninfectious inflammatory diseases of the cardiovascular system, lung and liver. TLR4 acts as receptor for LPS, a cell-wall component of Gram-negative bacteria that is among the strongest known inducers of inflammation. After liver injury, LPS levels increase in the portal and systemic circulation owing to changes in the intestinal mucosal permeability and increased bacterial translocation. Kupffer cells are the best-characterized target of LPS in the liver, where they have a crucial role in hepatic fibrogenesis by enhancing HSC activation. We and others have previously shown that activated HSCs express TLR4 and are highly responsive to low concentrations of LPS. Thus, TLR4 ligands may modulate hepatic fibrogenesis through two distinct targets, HSCs and Kupffer cells. Here we report that TLR4 and the intestinal microflora are essential for hepatic fibrogenesis, and that TLR4 downregulates the TGF-β pseudoreceptor BMP and the activin membrane-bound inhibitor (Bambi) on quiescent HSCs to augment TGF-β–mediated HSC activation and collagen production.

RESULTS

TLR4 enhances hepatic inflammation and fibrogenesis

To determine whether TLR4 is involved in hepatic fibrogenesis, we performed bile duct ligation (BDL) on TLR4-mutant mice (C3H/HeJ) and on TLR4-wild-type mice (C3H/HeOuJ). Twenty-one days after BDL, TLR4-wild-type mice showed overt hepatic fibrosis, whereas TLR4-mutant mice had a significant reduction in fibrosis as demonstrated by Sirius red staining, hydroxyproline content and expression of α-smooth muscle actin (α-SMA, encoded by Acta2) (Fig. 1a–d). In TLR4-mutant mice, a strong suppression of hepatic fibrogenesis was already present 5 d after BDL, as indicated by the reduction in messenger RNA expression of early markers of fibrogenesis including collagen-1 (encoded by Col1a1), Acta2, TGF-β1 (encoded by Tgfb1) and tissue inhibitor of metalloproteinases-1 (TIMP1, encoded by Timp1) (Fig. 1e). We observed a similar reduction in BDL-induced hepatic fibrosis, 5 and 21 d after BDL, in mice Tlr4-mutant from another genetic background (data not shown). Liver injury was not different between Tlr4–wild-type and Tlr4-mutant mice as assessed by measuring serum ALT levels (Fig. 1f). Next, we examined the recruitment of macrophages, a cell population that is required for HSC activation and hepatic fibrosis. Tlr4-mutant mice showed significantly reduced hepatic macrophage

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infiltration in comparison to Tlr4–wild-type mice (Fig. 1h). Additionally, the expression of Ccl2 and Ccl4, two potent mediators of macrophage recruitment, was decreased in Tlr4–mutant mice (Fig. 1h). To determine whether TLR4 activation is a universal requirement for hepatic fibrosis, we assessed fibrogenesis in two other, chemically-induced models of fibrosis, CCl4 and thioacetamide (TAA). Tlr4–mutant mice showed a significant reduction of hepatic fibrosis after eight doses of CCl4 or 20 weeks of TAA treatment (Supplementary Fig. 1a,b online). In all three models of fibrogenesis, plasma concentrations of the TLR4 ligand LPS were elevated (Supplementary Fig. 1c–f). In contrast with TLR4, the presence of TLR2, the main receptor for Gram-positive bacterial cell wall components3, was not required for hepatic fibrosis after BDL (Supplementary Fig. 2 online).

Gut-derived bacterial products mediate hepatic fibrosis

To investigate whether the intestinal bacterial microflora contributes to elevated LPS levels and to hepatic fibrosis, we treated mice with a cocktail of non-absorbable broad-spectrum antibiotics17 and then performed BDL. This antibiotic cocktail efficiently suppressed the increase in plasma LPS after BDL (Supplementary Fig. 1f). Mice receiving this cocktail showed a significant reduction in hepatic fibrosis as shown by decreased Sirius red staining, hydroxyproline content and Acta2 expression (Fig. 2a–e). Moreover, hepatic macrophage infiltration was suppressed (Fig. 2f,g), suggesting that intestinally derived TLR ligands mediate macrophage recruitment to the liver. This is further supported by the observed decrease in Tlr4 expression in antibiotic-treated mice (Fig. 2h), as Kupffer cells represent the most abundant source of Tlr4 mRNA in the liver. The antibiotic cocktail did not affect Tlr4 expression or the number of Kupffer cells in the liver before BDL (Fig. 2g,h), and mice treated with this cocktail showed a normal response toward exogenous LPS, thus excluding any effects of antibiotics on LPS signaling molecules and other downstream mediators (data not shown).

HSCs, but not Kupffer cells, promote TLR4-dependent fibrosis

Kupffer cells are the main targets of LPS in the liver, and they have a pivotal role in HSC activation and fibrogenesis10,12. To explore whether Kupffer cells mediate TLR4-dependent profibrogenic effects, we generated TLR4-chimeric mice using a combination of clodronate-mediated Kupffer cell depletion, irradiation and bone-marrow transplantation (BMT). This protocol achieved full reconstitution of Kupffer cells, whereas HSCs were not replaced by bone-marrow–derived cells (Fig. 3a). We confirmed successful BMT in all mice by assessing expression of Il6, the gene encoding interleukin (IL)-6, in LPS-stimulated spleen cells (Fig. 3b). To our surprise, chimeric mice containing Tlr4–mutant bone marrow showed normal fibrogenesis, whereas chimeric mice containing Tlr4–wild-type bone marrow but Tlr4–mutant resident liver cells showed the same reduction in fibrosis as Tlr4–mutant mice transplanted with Tlr4–mutant bone marrow (Fig. 3c–f). These results indicate that bone marrow–derived cells, including Kupffer cells, are not the primary cells that enhance hepatic fibrogenesis in response to TLR4 ligands. To test the hypothesis that TLR4 ligands directly target HSCs to mediate hepatic fibrogenesis, we first examined whether HSCs are targets of LPS in vivo. After intraperitoneally injecting LPS, we detected nuclear translocation of p65 in HSCs and hepatocytes (Fig. 4a). Given that Kupffer cells rapidly release large amounts of TNF-α in response to LPS13, the observed activation of NF-κB in HSCs and hepatocytes could not be directly attributed to LPS. To avoid the confounding effect of TNF-α, we were depleted of Kupffer cells by liposomal clodronate and were then injected with LPS. In Kupffer-cell–depleted mice, we still detected nuclear translocation of p65 in a large number of HSCs, whereas there was almost no p65 translocation in hepatocytes and in desmin-negative nonparenchymal cells (Fig. 4a). Next, we investigated TLR4 expression in freshly isolated HSCs. HSCs expressed large amounts of TLR4 in the quiescent state, and TLR4 expression in vivo was not further enhanced by activation (Fig. 4b). To further confirm that quiescent HSCs are targets of
Figure 2 Gut sterilization prevents hepatic fibrosis after bile-duct ligation. Tlr4−/−wild-type C3H/HeJ mice were gut-sterilized for 4 weeks before BDL and 3 weeks after BDL (n = 8) or underwent BDL without gut sterilization (n = 8). (a,b) Fibrillar collagen deposition was evaluated by Sirius red staining (a) and determination of the Sirius red–positive area (b) 21 d after BDL. Scale bar, 50 μm. *P < 0.001. (c) Hepatic hydroxyproline content was measured 21 d after BDL. *P = 0.035. (d,e) Expression of α-SMA was determined by immunohistochemistry (d) and western blotting (e) 21 d after BDL. Numbers in e indicate individual mice. Scale bar, 50 μm. (f,g) Macrophage infiltration as determined by immunohistochemical staining of F4/80 (f) and quantification by counting five randomly chosen high-power fields (g). IU, international units. *P = 0.016. Scale bar, 25 μm. (h) Tlr4 mRNA was quantified by qPCR in sham- or BDL-operated antibiotic-treated mice and sham- or BDL-operated control mice.*P = 0.048.

TLR4 enhances the interaction between HSC and Kupffer cells

We identified a large number of chemokines that were significantly upregulated in LPS-stimulated quiescent HSCs (Fig. 4d and Supplementary Table 1). Because Tlr4−/−mutant mice showed decreased recruitment of macrophages after BDL (Fig. 1g), we investigated whether stimulating HSCs with LPS induces Kupffer cell migration. Conditioned media from LPS-stimulated quiescent HSCs increased migration of Kupffer cells 2.5-fold in comparison to conditioned media from unstimulated HSCs (Fig. 4e). Kupffer cells from Tlr4−/−mutant mice induced approximately the same amount of chemotaxis as Kupffer cells from Tlr4−/−wild-type mice in this assay, demonstrating that LPS mediates its effects through HSCs and not through Kupffer cells. Additionally, we found upregulation of mRNAs encoding the adhesion molecules Icam1, Vcam1 and Sele in LPS-stimulated HSCs and increased adhesion of Tlr4−/−wild-type as well as Tlr4−/−mutant Kupffer cells to LPS-stimulated quiescent HSCs (Fig. 4f,g). To confirm the pivotal role of Kupffer cells in HSC activation and fibrosis in vivo, we depleted Kupffer cells by liposomal clodronate and then performed BDL. Liposomal clodronate achieved complete depletion of Kupffer cells (Supplementary Fig. 3a online) and had no direct effects on HSCs as assessed by transcription factor (NF-κB and Smad) reporter assays and an HSC activation assay (data not shown). After BDL, Kupffer cell–depleted mice showed an almost complete suppression of HSC activation and fibrosis (Supplementary Fig. 3b–g).

TLR4 enhances HSC activation induced by TGF-β or Kupffer cells

To explore the mechanisms by which TLR4 contributes to hepatic fibrogenesis, we investigated whether LPS promotes the transdifferentiation of quiescent HSCs to extracellular matrix–producing myofibroblasts by using a well-characterized reporter mouse that expresses GFP under control of the collagen-2α1 promoter (Coll-GFP)19. LPS treatment alone did not induce GFP expression or a myofibroblast-like phenotype in HSCs isolated from Coll-GFP mice (Fig. 5a). However, when quiescent HSCs were pretreated with LPS (100 ng/ml for 24 h), complete depletion of Kupffer cells (Supplementary Fig. 3a online) and had no direct effects on HSCs as assessed by transcription factor (NF-κB and Smad) reporter assays and an HSC activation assay (data not shown). After BDL, Kupffer cell–depleted mice showed an almost complete suppression of HSC activation and fibrosis (Supplementary Fig. 3b–g).
the response to the profibrogenic cytokine TGF-β1 was strongly enhanced, as shown by threefold higher GFP expression (Fig. 5a).

The enhanced response to TGF-β1 in LPS-pretreated HSCs was confirmed by a reporter assay in which luciferase expression is driven by Smad-binding CAGa elements (Fig. 5b), and by quantitative real-time PCR (qPCR) amplification of Cxcl1 mRNA (data not shown).

We also investigated whether LPS promotes fibrosis by sensitizing HSCs to platelet-derived growth factor (PDGF), another key player in HSC activation and fibrogenesis. However, we found neither LPS-mediated sensitization to PDGF-induced phosphorylation of the kinases Akt and Erk, nor increased PDGF-induced [3H]thymidine uptake in LPS-treated HSCs (Supplementary Fig. 4a,b online).

As Kupffer cells are an important source of TGF-β in the fibrotic liver, we investigated whether LPS pretreatment primes HSCs to Kupffer cell–induced activation. To exclude the effects of LPS on Kupffer cells in these experiments, we cultured HSCs from Coll-GFP mice with Tlr4–wild-type as well as Tlr4–mutant Kupffer cells. Under both conditions, LPS pretreatment strongly enhanced GFP expression, confirming HSCs as LPS targets in this assay (Fig. 5c).

Kupffer cell–induced GFP expression was completely blocked by soluble type II TGF-β receptor in LPS- and vehicle-treated HSCs, confirming that LPS-mediated enhancement of HSC activation requires TGF-β (Fig. 5c).

**TLR4-mediated Bambi downregulation sensitizes HSCs to TGF-β**

The TGF-β pseudoreceptor Bambi²⁰ was the only TGF-β–related gene among 121 LPS-regulated genes that we identified by microarray analysis in quiescent HSCs (Supplementary Table 1). Microarray analysis, qPCR and immunoblotting showed a strong downregulation of Bambi mRNA and Bambi protein levels in HSCs after LPS stimulation (Supplementary Table 1 and Fig. 5d). Notably, we also observed a significant downregulation of Bambi in HSCs isolated from Tlr4–wild-type mice after BDL (Fig. 5e) or Ccl3 treatment (data not shown), but not in those from Tlr4–mutant mice, suggesting that TLR4-dependent downregulation of Bambi is an integral part of the HSC activation process in vivo. LPS also decreased the activity of a Bambi-driven luciferase reporter (data not shown). The levels of several other TGF-β signaling molecules, including Smad2, Smad3, Smad4, Smad7, Sar1a, Skil, Sbe, Tgfrb1, Tgfrb2 and Tgfrb3, were not significantly altered by LPS treatment of quiescent HSCs, as assessed by microarray experiments (data not shown) and qPCR (Supplementary Fig. 4c). Moreover, LPS was at least as efficient in sensitizing HSCs to active TGF-β as in sensitizing them to latent TGF-β, a result that excludes any effects of LPS on TGF-β activation (Supplementary Fig. 4d). To explore the potential role of Bambi in mediating the TGF-β–sensitizing effects of LPS, we infected quiescent HSCs with adenoviruses expressing dominant-negative Bambi (AdBambi), full-length Bambi (AdBambi) or GFP (AdGFP), and measured Smad-driven luciferase activity after stimulation with LPS and TGF-β1. In uninfected and AdGFP-infected HSCs, LPS pretreatment enhanced TGF-β–induced Smad reporter activity 2.5-fold (Fig. 5f). AdBambi-infected cells showed a significant increase in Smad-driven luciferase activity, which was similar to that of LPS-pretreated, AdGFP-infected HSCs (Fig. 5f). Notably, TGF-β–induced luciferase activity could not be further enhanced by LPS in AdBambi-infected HSCs, suggesting that Bambi downregulation is the major mechanism by which LPS sensitizes HSCs to TGF-β–induced signals. Conversely, Smad reporter activity induced by TGF-β1 or TGF-β1 plus LPS was blunted in AdBambi-infected HSCs. In addition, we investigated whether Bambi altered TGF-β–induced HSC activation by using collagen-driven GFP expression as a marker of activation. AdBambi- or AdBambi-infected HSCs showed increased or reduced GFP expression, respectively, in comparison to HSCs infected with AdShuttle control virus (Fig. 5g). We next wanted to assess the effects of modulating Bambi activity in vivo with an adenoviral approach. To exclude any confounding effects of overexpressing dominant-negative Bambi in hepatocytes and Kupffer cells,
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**Figure 5** LPS downregulates Bambi to enhance TGF-β signaling and HSC activation. (a) Quiescent HSCs from Coll-GFP mice were treated with LPS (100 ng/ml) or vehicle for 24 h, and this was followed by TGF-β (100 pg/ml) or vehicle treatment for an additional 48 h. GFP expression was visualized by fluorescent microscopy (left) and quantified in six high-power fields (right). Scale bar, 25 μm. *P = 0.0002. (b) Quiescent HSCs were transduced with CAGA-luciferase and then treated with LPS (100 ng/ml) for 24 h and TGF-β (100 pg/ml) for 8 h. CAGA-driven luciferase activity is expressed as fold induction after normalization to β-galactosidase activity. *P = 0.003. (c) Quiescent HSCs from Coll-GFP mice were treated with LPS (100 ng/ml) or vehicle for 24 h and were then co-cultured with Kupffer cells in the presence or absence of soluble TGF-β receptor type II (sTRII) for an additional 48 h. Scale bar, 25 μm. *P = 0.003, **P = 0.002, ***P ≤ 0.02. (d–e) Bambi protein expression (top) and mRNA expression (bottom) were measured by western blotting and qPCR in untreated and LPS-treated (100 ng/ml for 6 h) quiescent HSCs (d) and in HSCs isolated from TLR4-wild-type and TLR4-mutant mice that had undergone BDL or sham operation for 2 weeks (e). *P = 0.03, **P = 0.006. (f) Quiescent HSCs were infected with CAGA-luciferase (multiplicity of infection (MOI) of 1000), β-galactosidase (MOI of 100) and either AddnBambi, AdBambi or AdGFP (both with an MOI of 300), and this was followed by TGF-β (100 pg/ml) treatment. CAGA-driven luciferase activity is expressed as fold induction after normalization to β-galactosidase activity. *P = 0.007, **P = 0.002, ***P = 0.001. (g) Quiescent HSCs from Coll-GFP mice were infected with AddnBambi or AdBambi (MOI of 300), pretreated with LPS (100 ng/ml) for 24 h and then treated with TGF-β (100 pg/ml) for an additional 48 h. Scale bar, 25 μm. *P < 0.001. All figures are representative of at least three independent experiments. dn, dominant negative.

**DISCUSSION**

Chronic hepatic inflammation is tightly linked to fibrosis in virtually all individuals with liver disease and in experimental models of fibrogenesis. Whereas chronic activation of inflammatory pathways has been shown to promote hepatocarcinogenesis22,23, the molecular link between inflammation and hepatic fibrogenesis remains elusive. Here we demonstrate that TLR4 drives myofibroblast activation and fibrogenesis in the liver and that TLR4-dependent modulation of TGF-β signaling provides a link between proinflammatory and profibrogenic signals. Tlr4-deficient mice show markedly decreased fibrogenesis in three different models of experimental hepatic fibrosis, and LPS sensitizes HSCs to TGF-β-induced signals through a Myd88-NF-κB pathway.
Figure 6 LPS downregulates Bambi through Myd88-dependent signals. (a) Quiescent HSCs isolated from wild-type, Myd88-deficient and Triflps2/lps2 mice were cultured for 6 h and then stimulated with LPS (100 ng/ml) for 6 h. Bambi expression was analyzed by qPCR. (b) Myd88-deficient and wild-type littersmates underwent BDL or sham operation for 5 d. Levels of Coll1a1, Acta2, Tnf, TGFβ1, TIMP1 and Il6 mRNA were determined by qPCR. *P ≤ 0.03, **P = 0.003, ***P = 0.047. (c) Triflps2/lps2 and C57BL/6 control mice underwent BDL or sham operation for 5 d. Levels of Coll1a1, Acta2, Tnf, TGFβ1, TIMP1 and Il6 mRNA were determined by qPCR. *P = 0.007. (d,e) Myd88-deficient and wild-type littersmates underwent BDL or sham operation. On the eighteenth day after BDL vs sham, tissue from Myd88-deficient and wild-type littersmates (d) and Triflps2/lps2 and C57BL/6 control mice (e) were tested for fibrillar collagen by Sirius red staining (left) and then quantified (right). Scale bar, 50 μm. WT, wild-type. *P < 0.001, **P = 0.014.

Although Kupffer cells are considered to be the main targets of TLR4 ligands in the liver, our study provides several lines of evidence that HSCs, and not Kupffer cells, are the primary targets that drive fibrogenesis in response to TLR4 ligands. First, chimeric mice containing Tlr4–mutant Kupffer cells and Tlr4–wild-type HSCs show normal fibrogenesis, whereas chimeric mice containing Tlr4–mutant HSCs and Tlr4–wild-type Kupffer cells show a similar reduction in fibrogenesis as Tlr4–mutant mice transplanted with Tlr4–mutant bone marrow. Second, quiescent HSCs express high levels of TLR4 and are highly responsive to LPS in vitro and in vivo. Lastly, LPS sensitizes HSCs to TGF-β- and Kupffer cell–mediated activation. Other cell populations, including hepatocytes, biliary epithelial cells and endothelial cells, are known to express TLR4 (ref. 10) but do not show a strong activation of NF-κB after LPS injection in vivo, which is consistent with previous reports24.

The liver is a main target of intestinally-derived bacterial products, and the rate of bacterial translocation increases in various models of hepatic disease, rendering LPS a likely candidate mediator of TLR4-dependent profibrogenic effects. Accordingly, we found increased amounts of LPS in all three models of fibrogenesis. Moreover, LPS levels and fibrogenesis were strongly reduced in mice that were treated with a combination of three nonabsorbable oral antibiotics and metronidazole, suggesting that the intestinal flora is the main source of LPS and that intestinally derived LPS drives fibrogenesis. Recently, it has been suggested that TLR4 is also activated by endogenous ligands such as HMGB1 and hyaluronan25–28. Although hepatic HMGB1 and hyaluronan were upregulated after BDL (data not shown), we found almost the same reduction of hepatic fibrosis in gut-sterilized mice as in Tlr4–mutant mice. Moreover, a blocking HMGB1 antibody did not reduce hepatic fibrogenesis after BDL (R.F.S., unpublished observations), suggesting that intestinally derived bacterial products are the predominant profibrogenic TLR4 agonists in hepatic fibrosis. However, we cannot completely exclude a contribution of endogenous TLR4 ligands to hepatic fibrogenesis, for example at other time points or in different models of liver injury. Our hypothesis that gut-derived LPS is an important mediator of hepatic fibrogenesis is further supported by a recent study that has demonstrated a reduction in hepatic fibrogenesis in CD14-deficient and LPS-binding protein (LBP)–deficient mice29. In contrast to our study, the authors conclude that Kupffer cells are the LPS targets that enhance hepatic fibrogenesis. In their study, the suppression of fibrogenesis was not as pronounced as the one we observed in Tlr4–mutant mice. This difference is most probably due to CD14- and LBP-independent effects of LPS. We propose that the increase in intestinal permeability and the rise of portal and systemic LPS levels after liver injury act as danger signals to prime HSCs for activation and to prepare the liver for wound-healing responses. Although these signals may be important for the response to acute injury, our results suggest that LPS-mediated TLR4 activation contributes to the excessive activation of wound-healing responses that is characteristic of chronic liver injury and that leads to the development of liver cirrhosis and its serious consequences in humans. Further investigations in germ-free and mono-associated animals are required to reveal how individual components of the intestinal microbiota contribute to hepatic fibrogenesis.

Our study defines HSC-mediated Kupffer-cell chemotaxis and sensitization to TGF-β–induced signals as two independent, yet complementary, mechanisms by which TLR4 enhances HSC activation and hepatic fibrosis. TGF-β is considered the most powerful mediator of HSC activation in vitro and in vivo1,2. Kupffer cells are a main source of TGF-β in the liver and promote HSC activation and fibrogenesis12,13,30. Thus, TLR4 mediates HSC activation through two TGF-β–dependent mechanisms: increased exposure to Kupffer cell–derived TGF-β and enhanced sensitivity to TGF-β. The importance of HSC-mediated Kupffer cell chemotaxis is emphasized by our...
finding that HSC activation and fibrogenesis were almost completely suppressed in Kupffer cell–depleted mice and by our observation that Ccr1–, Ccr2– and Ccr5–deficient mice show a strong suppression of hepatic Kupffer cell recruitment and a significant reduction of hepatic fibrogenesis (E.S. and R.F.S., unpublished data). Because the transdifferentiation of quiescent HSCs to activated myofibroblast-like HSCs is a key event in hepatic fibrogenesis1,2, it is most likely that the sensitization of quiescent HSCs to TGF-β– and Kupffer cell–induced activation constitutes a main mechanism by which TLR4 promotes fibrogenesis. However, we cannot exclude the possibility that TLR4 enhances hepatic fibrogenesis by additional mechanisms—perhaps by inducing proinflammatory pathways in activated HSCs, for example,3,4 or by upregulating antiapoptotic mediators such as Birc2 and Birc3 in HSCs (Supplementary Table 1). This notion is further supported by our recent finding that the presence of LPS modulates the unphysiological gene-expression pattern of culture-activated HSCs toward the pattern that is observed in vivo activated HSCs31.

Microarray analysis revealed that the TGF-β–pseudoreceptor Bambi is a target of LPS in quiescent HSCs. Bambi is a TGF-β family type I receptor that lacks an intracellular kinase domain and blocks signal transduction after stimulation with ligands of the TGF-β superfamily such as TGF-β, BMPs and activin30,32,34. Bambi is upregulated in hepatocellular carcinoma and inhibits TGF-β signaling in cancer, but its role in non-transformed hepatic cell populations is not known32,34. The greatly reduced abundance of Bambi in both LPS-treated quiescent HSCs and in vivo–activated HSCs from Tbr4–wild-type, but not Tbr4–mutant, mice strongly suggests that TLR4–mediated downregulation of Bambi is an integral part of HSC activation. TGF-β1– and Kupffer cell–mediated HSC activation is enhanced by LPS pretreatment or by overexpression of dominant-negative Bambi in quiescent HSCs, suggesting that downregulation of Bambi allows Kupffer cells to fully activate HSCs in a TGF-β–dependent manner. Thus, the downregulation of Bambi and the induction of Kupffer cell–attracting chemokines by LPS complement each other to enhance TGF-β–dependent HSC activation by Kupffer cells. Adenoviral overexpression of dominant-negative Bambi enhanced hepatic fibrosis, but because hepatocytes and Kupffer cells do not express Bambi, the effects of adenoviral dominant-negative Bambi on Bambi–TGF-β signaling in these cell populations are largely excluded. The moderate increase in hepatic fibrosis is explained by the strong downregulation of Bambi during HSC activation in vivo and the resulting restriction of dominant negative Bambi effects to early stages of fibrogenesis. Notably, we even observed an upregulation of HSC activation in AddnBambi–infected mice in the absence of BDL, suggesting that Bambi may act as a gatekeeper of HSC activation. Previous studies have shown that Bambi expression is regulated by Smad and β-catenin34,35; but these pathways are not directly activated by TLR4. We now present data demonstrating that Bambi expression is regulated through a MyD88–NF-κB–dependent pathway in HSCs. Our results suggest a role for the NF-κB pathway in regulating fibrogenic responses in the liver. This finding is consistent with a recent study revealing decreased hepatic fibrogenesis after NF-κB inhibition36. Although Trif–deficient mice show no alterations in LPS–mediated Bambi regulation in HSCs and have normal fibrogenesis after five days of BDL, they have less severe histologic fibrosis three weeks after BDL. Thus, it seems that TLR4 affects fibrosis through several mechanisms and that Trif–dependent signals are important only in later stages of fibrogenesis, independently of Bambi.

TLRs fulfill a variety of functions in the liver, and inhibition of TLR4 signaling may alter not only fibrogenesis but also biological processes not directly related to fibrogenesis10. Whereas TLR4 promotes disease progression in alcoholic and nonalcoholic steatohepatitis8,10,37,38, TLR4 activation may be beneficial in chronic hepatitis B virus infection, owing to TLR4–mediated reduction of hepatitis B virus replication24. In chronic hepatitis C virus infection, TLR4 is upregulated, but the determination of its precise role requires further investigation10. We and others have shown that liver regeneration is not altered in TLR4–deficient mice9,39, suggesting that a decrease of functional liver mass by TLR4 antagonism is unlikely. There are several TLR4 single-nucleotide polymorphisms (SNPs) that render humans less responsive to LPS. Notably, a recent study has identified the TLR4 SNP that results in a T399I change as a factor that confers a significantly reduced risk for fibrosis progression in patients with chronic hepatitis C virus infection40. Thus, TLR4 inhibition appears to be a promising strategy for the prevention of hepatic fibrosis in patients with chronic liver disease. Preventing LPS release from the intestinal flora may represent an additional strategy to inhibit hepatic fibrogenesis. Moreover, it is likely that the TLR4–Bambi–TGF-β signaling pathway plays a role in other cell types and affects wound-healing responses in organs such as the lung, kidney and heart.

**METHODS**

**Mice and fibrosis induction.** Specific pathogen–free 8-week–old male TLR4–wild-type (C3H/HeOu), TLR4–mutant (C3H/HeJ), Tbr2–deficient, Trif–deficient (ref. 41) and C57BL/6 mice (all from Jackson Laboratories) and MyD88–deficient mice42 underwent BDL, or were given CCl4 or TAA (see Supplementary Methods online). We anesthetized mice with ketamine and xylazine. After midline laparotomy, we ligated the common bile duct twice with 6–0 silk sutures and closed the abdomen. We performed the sham operation similarly, except that the bile duct was not ligated. We killed the mice 5 or 21 d after BDL. We gave the mice humane care according to US National Institutes of Health recommendations outlined in the “Guide for the Care and Use of Laboratory Animals”. All animal experiments were approved by the Columbia University Institutional Animal Care and Use Committee.

**Gut sterilization.** Mice were treated with ampicillin (1 g/l; Sigma), neomycin sulfate (1 g/l; Sigma), metronidazole (1 g/l; Sigma) and vancomycin (500 mg/l; Abbott Labs) in drinking water for 4 weeks. This was followed by BDL and continuation of antibiotic treatment until mice were killed43.

**Bone marrow transplantation.** We performed BMT experiments as previously described with slight modifications44. Because only 30% of Kupffer cells are reconstituted by donor–derived bone marrow cells 6 months after BMT44, we gave mice an intravenous injection of liposomal clodronate (200 µl intravenously) before irradiation to deplete Kupffer cells and accelerate macrophage turnover45. We then flushed the thymus and femurs of donor mice to obtain bone marrow. We washed bone marrow cells twice in HBSS and injected 1 × 107 bone marrow cells into the tail veins of lethally irradiated (11 Gy) recipient mice. We performed BDL 12 weeks after BMT and checked the efficiency of reconstitution by transplanting wild-type mice with bone marrow from mice expressing transgenic GFP from the β-actin promoter43, as described above. To determine successful BMT in TLR4–mutant and TLR4–wild–type mice, spleen cells were isolated from bile duct–ligated chimeric mice, then stimulated with LPS (100 ng/ml) and analyzed by qPCR to measure induction of Il6 mRNA expression.

**HSC and Kupffer cell isolation and culture.** We isolated quiescent HSCs by a two-step collagenase–pronase perfusion of mouse livers followed by 8.2% Nycodenz (Accurate Chemical and Scientific Corporation) two–layer discontinuous density gradient centrifugation as previously described46,47. To ensure the highest possible purity, we depleted HSCs of Kupffer cells and macrophages by magnetic antibody cell sorting (MACS, Miltenyi Biotech) with antibody to F4/80 antigen (ebioscience) and CD11b–conjugated microbeads (Miltenyi Biotech). We used the same procedure to isolate in vivo–activated HSCs from mice that had undergone BDL for 2 weeks. To isolate Kupffer cells,
we performed collagenase-pronase perfusion followed by 15% Nycodenz gradient centrifugation and subsequent positive selection of F4/80-expressing cells by MACS11. The described procedures resulted in 99% purity of HSCs and 95% purity of Kupffer cells, as judged by retinoid autofluorescence and flow-cytometric analysis for F4/80 expression, respectively. We cultured HSCs on uncoated plastic tissue culture dishes in DMEM containing 1% or 10% FBS used them as non-passaged primary cultures only. Murine Kupffer cells were not passaged and were cultured in DMEM containing 1% FBS.

**Immunohistochemistry.** For immunohistochemical analysis, we fixed liver specimens in 10% buffered formalin and incubated them with monoclonal antibody to α-SMA (clone 1A4, DakoCytomation) or monoclonal antibody to F4/80 (clone BM8; eBioscience) using the MOM kit (Vector Laboratories). For immunofluorescent staining, we fixed liver specimens with 4% paraformaldehyde, incubated them in PBS containing 30% sucrose and froze them at −80 °C. We then incubated them with antibody to F4/80, antibody to desmin and antibody to p65 (Santa Cruz Biotechnology) and imaged them with confocal microscopy.

**Western blot.** We performed electrophoresis of protein extracts and subsequent blotting as described.6 We incubated blots with mouse antibody to α-SMA (Sigma), antibodies to mouse Bambi (R&D Systems), phospho-Erk, total Erk, phospho-Akt (on Ser473), total Erk (all from Cell Signaling Technologies, Inc.) or HMGB1 (R&D Systems) at a dilution of 1:500–1:5000 and visualized them by the enhanced chemiluminescence light method (Amershams Biosciences). Blots were reprobed with mouse antibody to β-actin (Sigma).

**Measurement of collagen-driven green fluorescent protein expression in hepatic stellate cells.** To measure collagen-promoter activity, we isolated HSCs (1 × 10^6 cells/well) from collagen promoter-driven GFP transgenic mice19 (pColGFP-HS4.5 transgene) and incubated them with LPS (100 ng/ml) or vehicle for 24 h, and this was followed by treatment with TGF-B1 (300 pg/ml) for an additional 24 h. We determined the number of GFP-positive cells by counting GFP-positive cells and total cells in ten randomly chosen high-power fields. For co-culture experiments, we isolated HSCs (1 × 10^5 cells/well) from Coll-GFP–transgenic mice, cultured them in 24-well plates in the presence or absence of LPS (100 ng/ml for 24 h) and then co-cultured them with Kupffer cells (5 × 10^5 cells/well) from either Tlr4-wild-type or Tlr4-mutant mice for 48 h in the presence or absence of 20 ng/ml soluble type II TGF-β receptor (R&D Systems).

**Statistical analysis.** All data are expressed as mean ± s.d. Differences between experimental and control groups were assessed by two-tailed unpaired Student’s t-tests using SPSS 13.0 (SPSS, Inc.). P-values less than 0.05 were considered statistically significant.

**Additional methods.** Detailed methodology is described in Supplementary Methods.

**Note:** Supplementary information is available on the Nature Medicine website.

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**AUTHOR CONTRIBUTIONS**

E.S. performed animal surgeries and fibrosis evaluation, cell isolations, cell-culture studies, reporter assays and real-time PCR, generated recombinant adenoviruses, created figures and contributed to the design of the study and the writing of the manuscript. S.D.M. performed cell isolations, real-time PCR and microarray studies. Y.O. generated recombinant adenoviruses. J.K. performed animal surgeries and cell isolations, performed western blots and assisted in fibrosis evaluation and in manuscript preparation. D.A.B. contributed to the design of the study, experimental design and the writing of the manuscript. R.F.S. (the principal investigator) designed and coordinated the study, contributed to experimental setup, data analysis and interpretation, and drafted and edited the manuscript.

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