Endothelial Cell Toll-Like Receptor 4 Regulates Fibrosis-Associated Angiogenesis in the Liver

Kumaravelu Jagavelu,* Chittaranjan Routray,* Uday Shergill, Steven P. O’Hara, William Faubion, and Vijay H. Shah

Angiogenesis defines the growth of new blood vessels from preexisting vascular endothelial networks and corresponds to the wound healing process that is typified by the process of liver fibrosis. Liver fibrosis is also associated with increased endotoxin within the gut lumen and its associated portal circulation. However, the interrelationship of gut endotoxin and its receptor, toll-like receptor 4 (TLR4), with liver fibrosis and associated angiogenesis remains incompletely defined. Here, using complementary genetic, molecular, and pharmacological approaches, we provide evidence that the pattern recognition receptor that recognizes endotoxin, TLR4, which is expressed on liver endothelial cells (LECs), regulates angiogenic responses both in vitro and in vivo. Mechanistic studies have revealed a key role for a cognate TLR4 effector protein, myeloid differentiation protein 88 (MyD88), in this process, which culminates in extracellular protease production that regulates the invasive capacity of LECs, a key step in angiogenesis. Furthermore, TLR4-dependent angiogenesis in vivo corresponds to fibrosis in complementary liver models of fibrosis.

Conclusion: These studies provide evidence that the TLR4 pathway in LECs regulates angiogenesis through its MyD88 effector protein by regulating extracellular protease production and that this process is linked to the development of liver fibrosis. (HEPATOLOGY 2010;52:590-601)

Angiogenesis, the sprouting of new vessels from preexisting ones, is an essential physiological process required for embryogenesis, growth, regeneration, and wound healing.1 In liver cirrhosis, an exuberant wound healing response to liver injury culminates in fibrosis, angiogenesis, and vascular reorganization.2 However, the precise relationship between fibrosis, angiogenesis, and vascular reorganization has remained enigmatic.

Toll-like receptors (TLRs) belong to a class of pattern recognition receptors and bind molecules broadly shared by pathogens that collectively are called pathogen-associated molecular patterns.3,4 At least 10 mammalian TLRs have been cloned, and each recognizes a specific molecular product derived from major classes of pathogens.5 Within this family of TLR proteins, TLR4 recognizes lipopolysaccharide (LPS), a gram-negative bacterial cell wall component that is enriched

Abbreviations: 3D, three-dimensional; BDL, bile duct ligation; CCL4, carbon tetrachloride; cDNA, complementary DNA; CO2, carbon dioxide; DAPI, 4,6-diamidino-2-phenylindole; DN, dominant-negative; FGF, fibroblast growth factor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; H&E, hematoxylin and eosin; Hb, hemoglobin; HUVEC, human umbilical vein endothelial cell; LEC, liver endothelial cell; LPS, lipopolysaccharide; MLEC, murine liver endothelial cell; MMP, matrix metalloproteinase; mRNA, messenger RNA; MT, mutant; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; MyD88, myeloid differentiation protein 88; PCR, polymerase chain reaction; RT-PCR, real-time polymerase chain reaction; SEM, standard error of the mean; siRNA, short interfering RNA; TLR, toll-like receptor; TRAM, toll-like receptor adaptor molecule; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor; WT, wild-type; YFP, yellow fluorescent protein.

From the GI Research Unit and Fiterman Center for Digestive Diseases, Mayo Clinic, Rochester, MN.

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Address reprint requests to: Vijay H. Shah, M.D., GI Research Unit and Fiterman Center for Digestive Diseases, Mayo Clinic, Rochester, MN 55905. E-mail: shah.vijay@mayo.edu; fax: 507-255-6318.

*These authors contributed equally to this work.

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within the intestinal lumen and its associated portal circulation.

TLR4 maintains the ability to signal through the adapter molecule, myeloid differentiation protein 88 (MyD88), and an MyD88-independent pathway. In the canonical TLR4-MyD88 pathway, binding of TLR4 by LPS activates MyD88 through its cytosolic domain, which further triggers a cascade of intracellular signaling events leading to activation of nuclear factor kappa B and inflammation. Conversely, TLR4 stimulated the expression of interferon-β in a MyD88-independent fashion involving toll-like receptor adapter molecule (TRAM; also known as TIR domain-containing protein). Other noncanonical pathways have also been recently identified. Nonetheless, some recent reports have suggested that in vascular endothelial cells, TLR4 signals may channel preferentially through MyD88.

Previous studies have associated portal venous LPS with cirrhosis and suggested a possible direct effect of LPS on Kupffer cells and hepatic stellate cells. However, liver endothelial cells (LECs) are the first line of cells exposed to portal venous LPS. These cells also mediate sinusoidal remodeling and angiogenesis, processes that accompany liver fibrosis. These observations indicate a potential role of LPS in LEC signaling, and this is a compelling scenario.

On the basis of these concepts, we hypothesize that TLR4 signaling within LECs contributes to angiogenesis, sinusoidal remodeling, and cirrhosis. In support of this hypothesis, we demonstrate TLR4 expression and function in LECs leading to angiogenesis in vitro. Mechanistically, this effect is achieved by virtue of the TLR4 effector protein, Myd88, and culminates in secretion of the extracellular protease, matrix metalloproteinase 2 (MMP2), which promotes LEC invasion. Furthermore, angiogenesis and fibrosis are concurrently attenuated in TLR4-deficient mice. Lastly, we provide direct in vivo evidence that TLR4 mediates angiogenesis in complementary models of angiogenesis. Thus, these multidisciplinary studies expand our understanding of angiogenesis and its relationship with fibrosis and concurrently identify a new function for pattern recognition receptors in endothelial cells.

Materials and Methods

Animals. C3H/HeOuJ [TLR4-wild-type (WT)] mice and C3H/HeJ [TLR4-mutant (MT)] mice, which carry a spontaneous mutation that confers a loss of TLR4 function, were purchased from Jackson Laboratories (Bar Harbor, ME). These animals have similar levels of tumor necrosis factor-α under baseline conditions but impaired production in response to LPS. Bile duct ligation (BDL) and sham surgeries were performed as previously described. For carbon tetra-chloride (CCl4)-induced fibrosis studies, CCl4 (1 mg/kg of body weight) or vehicle (olive oil) was injected intraperitoneally for a period of 6 weeks as previously described. LECs were isolated from mice as previously described, and the purity was assessed (supplementary Fig-1). All procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Cell Culture and Transfection. Human LECs (Sci- enCell, San Diego, CA) were grown under standard tissue culture conditions [a humidified 5% carbon dioxide (CO2) incubator at 37°C] in media containing 5% fetal bovine serum, 2% endothelial cell growth supplement, and 1% penicillin/streptomycin (ScienCell). Retroviral transduction and short interfering RNA (siRNA) transfection were performed as we previously described. Two distinct siRNAs for TLR4 within the coding regions starting at 105 and 174 bp and MyD88 were gifts from Steven P. O’Hara, whereas TRAM siRNA was commercially obtained (Thermo Scientific). Human MyD88 full-length and dominant-negative N-terminal truncation MT constructs were polymerase chain reaction (PCR)–amplified from pUNO-hMyD88 and pDeNy-hMyD88 (InvivoGen), respectively, and the amplified fragments were subcloned into the pMMP retroviral vector.

In Vitro Tubulogenesis. Murine and human LECs, transduced with MyD88 or yellow fluorescent protein (YFP) retrovirus or preincubated with an MyD88 dimerization inhibitor (Imgenex) for 24 hours, were cultured in an endothelial cell medium before being plated into 4-well coated chamber slides (2 x 10⁶ cells per well) precoated with 70 μL of Matrigel (growth factor-reduced; BD Biosciences, San Jose, CA). Tubulogenesis was visualized with a Zeiss Axiovert 40 CFL inverted microscope (x4 magnification; Carl Zeiss, Ltd.), captured with a charge-coupled device digital camera (Jenoptik, Jena, Germany) after 3 or 6 hours of culturing in the presence of either vehicle or 1 μg/mL LPS at 37°C with 5% CO2, and quantified with Image Pro Software as previously described. Cell viability was measured with calcein AM (Invitrogen).

Matrigel Plug Assay. Anesthetized TLR4-WT and TLR4-MT mice received 300-μL injections of sterile Matrigel (growth factor reduced; catalog no. 356231, BD Biosciences) and vascular endothelial growth factor (VEGF; 50 ng/mL; R&D Systems, Minneapolis,
MN) into the subcutaneous layer in two locations. Matrigel plugs were removed 14 days after implantation, photographed, and divided into two blocks. One Matrigel plug was allowed to liquefy at 4°C, and the hemoglobin content was determined by the Drabkin method according to the manufacturer’s protocol (Sigma, St. Louis, MO). Absorbance was measured at 540 nm, and the hemoglobin concentration was calculated and normalized to the plug weight. The other Matrigel block was fixed overnight in formalin and embedded in paraffin. Sections (6 μm) were stained with hematoxylin and eosin (H&E) and were visualized by traditional light microscopy.

**Immunoblotting.** Proteins from cellular extracts were subjected to denaturing 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membranes. After blocking, the blots were probed with anti-TLR4 (1:1000) and anti-MyD88 (1:1000; Imgenex). The blots were washed and incubated for 1 hour at room temperature with appropriate horseradish peroxidase–conjugated secondary antibodies. Protein bands were detected with an enhanced chemiluminescence detection system (ECL Plus, Santa Cruz). After the nitrocellulose sheets were exposed to Kodak XAR film, the autoradiographs were scanned. Equal protein loading was verified by the reprobing of the membrane with an anti–β-actin antibody (1:5000).

**Confocal Immunofluorescence Microscopy.** For immunostaining, murine and human LECs were cultured to approximately 50% confluence on gelatin-coated cover slips in 24-well plates. Frozen liver sections from sham, BDL, olive oil–treated, and CCl4-treated TLR4-cover slips in 24-well plates. Frozen liver sections from WT and TLR4-MT mice were fixed with ice-cold acetone and were blocked with 10% goat serum for 2 hours at room temperature to eliminate nonspecific background signals. Cells or tissue sections were then incubated with antibodies against TLR4 (Sigma; 1:400), von Willebrand factor (vWF; Sigma; 1:400), F4/80 (Abcam; 1:150), CD11b (Abcam; 1:200), aquaporin-1 (Alpha Diagnostics International; 1:500), and platelet-derived growth factor receptor β (Cell Signalling; 1:100) at 4°C overnight (recent studies have shown that aquaporin-1 stains LECs, including cirrhotic neovessels). This was followed by incubation with appropriate fluorescein isothiocyanate or Alexa Fluor 568–conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature. Nuclei were counterstained with TOTO-3 stain. Immunofluorescent staining was visualized with a Zeiss LSM Pascal Axiovert confocal microscope (Carl Zeiss), and images from vWF and aquaporin-1 staining were quantified with Metamorph software (version 7.6, Molecular Devices, United States). Fibrosis quantification was carried out with Sirius red–stained sections.

**Aortic Ring Assay.** Aortas were excised from the thoracic region of 8-week-old male TLR4-WT or TLR4-MT mice and immediately placed in ice-cold phosphate-buffered saline. The fat tissue was removed atraumatically, and the aortas were subsequently cut into 0.3-mm rings with a dissecting microscope. The rings were then placed in 100 μL of Matrigel (growth factor reduced; catalog no. 356231, BD Biosciences) and incubated at 37°C in a humidified 5% CO2 incubator for 7 days. The rings were fixed in 4% formaldehyde; photographs of the rings were captured with a phase contrast microscope (Zeiss; ×10 magnification) and with a charge-coupled device camera (Jenoptix). Morphometric analysis of sprouting specifically within the vessel ring lumen was quantified with Image Pro software (Media Cybernetics, Bethesda, MD).

**Real-Time Polymerase Chain Reaction (RT-PCR).** Total RNA was extracted from human and mouse LECs with TRIzol (Invitrogen), and complementary DNA (cDNA) synthesis was performed with 1 μg of total RNA with SuperScript III (Invitrogen). Real-time amplification was carried out with Applied Biosystems 7500 detection systems. Species-specific primers were designed and used (sequences are available upon request). TLR4 messenger RNA (mRNA) levels were normalized to β-actin mRNA and were shown as fold changes.

**Transwell Collagen Invasion Assay.** LEC invasion was studied with a three-dimensional (3D) collagen assay as previously described. Polycarbonate membrane Transwell inserts (8-μm pore size; Corning, United States) were coated with collagen type I (50 μg/μL). Primary LECs from TLR4-WT or TLR4-MT mice were plated onto the membrane of the Transwell insert (40,000 cells/well) on top of a thick layer of Type I collagen (3 mg/mL). The lower chambers were filled with a serum-free medium containing 10 ng/mL mouse VEGF or fibroblast growth factor (FGF) or vehicle. Transwell inserts were removed after 24 hours of incubation, fixed, stained with 4′,6-diamidino-2-phenylindole (DAPI), and quantified with Metamorph Software (version 7.6, Molecular Devices).

**Gelatin Zymography.** Murine LEC isolates were lysed and separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis; the gel was impregnated with 1 mg/mL gelatin. The gel was then
renatured for 30 minutes in 2.5% Triton X-100 and subsequently incubated for 24 hours at 37°C in a substrate buffer (50 mmol/L tris(hydroxymethyl)aminomethane/hydrochloric acid (pH 7.5) containing 5 mmol/L calcium dichloride and 0.02% Brij-35) for MMP degradation of gelatin. Gels were stained with 0.5% Coomassie blue. Quantification of the gelatinolytic areas was measured with ImageJ (National Institutes of Health, Bethesda, MD).

**In Situ Zymography.** Frozen liver sections from TLR4-WT and TLR4-MT mice were incubated in 1% agarose fortified with fluorescent gelatin (Molecular Probes, Invitrogen). The sections were then incubated at 37°C in a substrate development buffer, and ethylene diamine tetraacetic acid was used as a negative control as previously described.25

**Proliferation Assay.** Primary LECs were cultured for 24 and 48 hours and subsequently incubated with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega, Madison, WI). The absorbance of the plate was read colorimetrically at an optical density of 490 nm. Standardization and other steps were performed according to the manufacturer’s instructions.

**Statistical Analysis.** Data are expressed as means and standard errors of the mean (SEMs) of at least three independent experiments. Groups were compared by a two-tailed Student t test. A P value less than 0.05 was considered statistically significant.

**Results**

**Expression of TLR4 in Human and Murine LECs.** As a first step in exploring a role for TLR4 in liver fibrosis–associated angiogenesis, we determined the expression of TLR4 in LECs from both humans and mice. Confirming prior studies,26 quantitative RT-PCR analysis detected TLR4 mRNA levels in both human and murine LECs; levels were substantially elevated in comparison with other systemic human endothelial cells such as human umbilical vein endothelial cells (HUVECs), although they were less elevated than the levels of lymphocyte-positive control Raji cells (Fig. 1A). This observation was substantiated by detection of a specific immunofluorescence signal for TLR4 in isolated mouse and human LECs (Fig. 1B); this indicated that TLR4 was expressed in both murine and human LECs. Although other TLR molecules were also expressed within LECs (data not shown), they were not pursued in further detail in this work. Instead, the present study was focused in a hypothesis-based manner on the recognition of TLR4 by LPS and its potential relevance to liver injury, fibrosis, and vascular integrity due to the proposed links of LPS with these processes.

**TLR4 Promotes LEC-Driven Angiogenesis In Vitro.** Reorganization of endothelial cells into tubelike vascular structures in Matrigel, which is called tubulogenesis, provides an in vitro estimation of the angiogenic capacity of vascular cells because a number of steps required for angiogenesis in vivo are required for tubulogenesis in vitro.15 To test TLR4 functional relevance in angiogenesis, we isolated LECs from TLR4-MT or TLR4-WT mice and measured tubulogenesis. As shown in Fig. 2A,B, although LPS prominently stimulated tubulogenesis in WT mice, both basal tubulogenesis and LPS-stimulated tubulogenesis were markedly attenuated in TLR4-MT mice. The antibiotic polymyxin-B inhibited tubulogenesis in all groups, and this further supports the role of basal LPS and TLR4 in this process.

To study the functional relevance of TLR4 in human cells, we next performed corroborative studies
with TLR4 siRNA. The increased tubulogenesis of human LECs in response to LPS is demonstrated in Fig. 2B. Transfection of TLR4 siRNA into human LECs significantly inhibited basal tubulogenesis in comparison with a scrambled siRNA control (Fig. 2C,D; P < 0.05; the inset western blot depicts siRNA knockdown of the doublet TLR4 protein band as previously described27). Additionally, the reduction in tubulogenesis was not due to cell toxicity; this was assessed by the staining of cells in Matrigel with the cell viability dye calcein AM (Supporting Fig. 2A,B). Similar results were also obtained with a second TLR4 siRNA recognizing a distinctly different region of human TLR4 mRNA (Fig. 2C). In these and ensuing in vitro experiments of tubulogenesis conducted on Matrigel, we observed prominent effects of experimental interventions on basal responses in the absence of LPS, and these were likely due to endogenous TLR4 ligands present within matrix-rich environments such as Matrigel.28-30 Therefore, the data are depicted as basal responses to Matrigel rather than the addition of exogenous LPS. These complementary genetic and molecular approaches provide evidence that TLR4 promotes angiogenesis in LECs in vitro.

**MyD88 Is Responsible for TLR4-Driven Tubulogenesis.** TLR4 signaling in response to LPS may occur by an MyD88-dependent or MyD88-independent, TRAM-dependent pathway.6 To identify the pathway that mediates the angiogenic signals of TLR4, we over-expressed MyD88 with a retroviral construct in human LECs. MyD88 overexpression in human LECs significantly enhanced tubulogenesis in comparison with cells transduced with a control retrovirus (Fig. 3A). To confirm specificity, we transfected human LECs with MyD88 siRNA or control siRNA. Basal tubulogenesis was reduced in LECs transfected with MyD88 siRNA.
in comparison with control siRNA (P < 0.05; Fig. 3B) in the absence of siRNA-induced cell toxicity (Supporting Fig. 2C,D). To further confirm whether TLR4-dependent angiogenesis occurs through MyD88 function, we blocked MyD88 homodimerization with the peptide IMG-2005-1 and thus blocked MyD88 function. The MyD88 inhibitory peptide attenuated tubulogenesis in human LECs in comparison with a vehicle control peptide (Fig. 3C). Furthermore, overexpression of a dominant-negative, N-terminal truncated form of MyD88 also significantly reduced tubulogenesis (Fig. 3D). To further link TLR4 signals through MyD88, we silenced MyD88 in human LECs and returned to the LPS stimulation model. Indeed, silencing of MyD88 reduced LPS-mediated tube formation in comparison with control siRNA, and this suggested that angiogenic signaling in these cells requires MyD88 activation downstream of TLR4 (Fig. 3E).

Conversely, a small and not statistically significant difference in tubulogenesis was observed through the silencing of TRAM with siRNA (Supporting Fig. 3). In all, these studies using multiple complementary approaches indicate that TLR4-dependent tubulogenesis is mediated through MyD88.

Matrigel Invasive Capacity Is Reduced in TLR4-MT Endothelial Cells. The ability of endothelial cells to invade through matrix is a key cellular step involved in angiogenesis, especially in the cirrhotic microenvironment. We were especially interested in potential effects of TLR4 on matrix regulatory proteins relevant for invasion because our initial hypothesis-generating, focused microarray analyses (endothelial cell superarray, SA Bioscience), comparing gene expression profiles of TLR4-WT and TLR4-MT LECs, revealed prominent differences in expression levels of several MMPs and tissue inhibitors of metalloproteinase (Supporting
Fig. 5A). To determine whether TLR4 regulates the matrix invasive capacity of LECs, primary murine LECs were plated onto Transwell chambers coated with collagen, and cell invasion was measured. TLR4-MT LECs evidenced reduced invasion (Fig. 4A,B) in response to VEGF or FGF in comparison with TLR4-WT LECs. However, no significant difference in the proliferation of primary LECs isolated from TLR4-WT or TLR4-MT mice at 24 and 48 hours was observed by the MTS proliferation assay, which provided a relevant control (Supporting Fig. 4). To assess the mechanism by which TLR4 may regulate LEC invasion, we measured the levels of MMP2, a key extracellular protease that promotes cell invasion and is highly relevant to cirrhosis, by gelatin zymography. Indeed, both active and pro forms of MMP2 were reduced in both cell lysates and supernatants of TLR4-MT LECs in comparison with TLR4-WT LECs (Fig. 4C,D; duplicate samples are depicted). Furthermore, TLR4-MT mouse livers evidenced reduced gelatinase activity in comparison with TLR4-WT mice according to in situ gelatin zymography (Supporting Fig. 5B), and this was consistent with previous studies showing that TLR4 regulates MMP production. These results suggest that reduced angiogenesis observed in TLR4-MT LECs may be due to reduced MMP2-dependent invasive capacity.

Angiogenesis Is Reduced in TLR4-MT Mice in Intact Tissues and In Vivo. Next, to directly determine if TLR4 regulates angiogenesis in vivo, we subcutaneously injected Matrigel into TLR4-WT and TLR4-MT mice. TLR4-MT mice showed significantly reduced neovascularization in comparison with TLR4-WT both grossly and histologically (Fig. 5A,B). To further confirm reduced neovascularization, we quantified the hemoglobin content of the Matrigel plug, which was also significantly reduced in TLR4-MT mice in comparison with TLR4-WT mice (Fig. 5C). These results were also extended to an additional model of angiogenesis, the aortic ring assay, in which
aortas from TLR4-WT and TLR4-MT mice were sectioned and cultured in vitro. Vascular sprout formation from the rings was measured as a parameter of angiogenic potential. In line with the previous vascular analyses, aortic rings derived from TLR4-MT mice showed less sprouting when stimulated with LPS in comparison with WT aortic rings (Fig. 5D), and this further corroborated an angiogenic role for endothelial cell TLR4.

**TLR4 Deletion Shows Parallel Effects on Both Liver Fibrosis and Liver Angiogenesis.** Using two distinct models of liver injury and fibrosis, we next sought to examine the role of TLR4 in the angiogenic response that is associated with fibrosis. First, BDL was performed in TLR4-WT and TLR4-MT mice, which were sacrificed after 3 weeks. Histological analysis revealed reduced fibrosis in TLR4-MT mice versus TLR4-WT mice (Fig. 6A,B; Sirius red and H&E, respectively), and this was consistent with recently published data. A more detailed analysis of the hepatic vasculature revealed that vWF-positive endothelial cell density was markedly increased in TLR4-WT mice after BDL in a manner that corresponded to the degree of liver fibrosis (Fig. 6C,D). Corroborative results were obtained with an additional endothelial cell marker, aquaporin-1 (Supporting Fig. 6). Furthermore, the diminished fibrosis that was observed in BDL TLR4-MT mice corresponded to diminished vascular density in these mice. Concordant results were also observed in TLR4-WT and TLR4-MT mice who underwent analysis after CCl₄-induced liver fibrosis, and they further substantiated the role of LEC TLR4 in fibrosis-associated angiogenesis (Fig. 7A,B depicts fibrosis as assessed by Sirius red staining, Fig. 7C,D depicts vascular density based on vWF-positive endothelial cell staining, and Supporting Fig. 6C,D depicts aquaporin-1–positive vascular density in CCl₄ mice).

**Discussion**

Because gut-derived LPS traverses directly into the liver via the portal vein, effects of the TLR4 pathway on liver function and pathobiology are an emerging area of interest. In turn, changes in vascular function...
and structure are increasingly recognized to be closely linked to liver injury and fibrosis. Our present work makes a number of important observations that link TLR4 to angiogenesis and liver fibrosis. Specifically, our study provides the following new findings: (1) TLR4 is expressed in LECs and contributes to cirrhosis-associated angiogenesis in liver, (2) TLR4 angiogenic signaling in LECs occurs through the MyD88-dependent pathway, (3) TLR4 angiogenesis is associated with MMP2-mediated LEC matrix invasion, and (4) inhibition of TLR4 inhibits angiogenesis in parallel with fibrosis in murine models of liver injury and cirrhosis and provides an important link between the two processes.

TLR4 is a pattern recognition molecule that detects specific proteins derived from bacteria, viruses, and fungi and therefore plays a key role in innate immunity. TLR4, in particular, detects LPS from the cell wall of gram-negative bacteria. Although most extensively studied in traditional blood immune cells, LPS binding to the endothelial cell surface may regulate endothelial cell immune function through the TLR4–myeloid differentiation 2–CD14 complex. Our study adds to the current paradigms of TLR4 function in endothelial cells by revealing that TLR4-induced activation of LECs leads to angiogenesis. Indeed, LECs from TLR4-MT mice revealed prominent defects in angiogenic function as revealed by a number of complementary in vivo and in vitro assays, including tubulogenesis, aortic sprouting, and Matrigel plug assays. Because MyD88 is an essential adaptor protein for TLR4 signaling, we postulated that TLR4 angiogenic signals may transduce through MyD88. Indeed, overexpression of MyD88 in LECs increased tubulogenesis, whereas inhibition of this pathway by siRNA-based silencing, dominant-negative perturbation, and small-
molecule inhibition blocked angiogenic signals. However, some of the quantitative differences in tubulogenesis that we observed in response to inhibition of the two pathways suggest that other noncanonical pathways could also be contributing. Thus, our work mechanistically builds on previous work pertaining to LPS and LEC function and also identifies links to cirrhosis pathobiology with mechanistic insights, as further outlined next.

VEGF expression is increased in the cirrhotic liver, and furthermore, vascular endothelial proliferation and vascular density are increased in both human and murine cirrhosis. Indeed, it has been postulated that active angiogenesis may perpetuate the fibrosis process through multiple potential mechanisms. In the BDL model, BDL causes portal hypertension and mesenteric congestion, which may promote translocation of LPS from intestinal microflora to the hepatic sinusoids across the gut barrier. Thus, we postulate that this endotoxic load may activate TLR4 in liver sinusoidal endothelial cells and thereby promote angiogenesis in conjunction with fibrosis. Indeed, we observed significant histological changes in TLR4-MT mice after BDL versus the WT and sham-operated controls, with immunohistochemistry revealing not only less fibrosis but also less neovascularization. Because concordant results were obtained in the mechanistically distinct CCl4 model, these observations in all suggest that TLR4 signaling in LECs may provide a requisite link between hepatic neovascularization and fibrogenesis. Indeed, this observation is of particular interest in the context of recent studies determining
that TLR4 in hepatic stellate cells is a key driver of the fibrosis process. Studies requiring the generation and utilization of mice with targeted deletion of TLR4 exclusively in LECs, Kupffer cells, or hepatic stellate cells will be required to elaborate further on the specific contribution of LEC TLR4 to the liver fibrosis process.

Endothelial cell invasion and matrix degradation are prerequisites for angiogenesis. In the 3D collagen invasion assay, we found reduced invasive capacity of TLR4-MT LECs, which was attributed to reduced MMP2 production. Although matrix constituents clearly influence sinusoidal cell behavior, precisely how endothelial cells sense the changes in matrix in their microenvironment is not well understood.

Because some studies suggest that TLR4 binds specific fibronectin splice variants, hyaluronan, and other matrix components in addition to its canonical LPS fibronectin splice variants, hyaluronan, and other matrix constituents in addition to its canonical LPS binding capacity, it is tempting to speculate that TLR4 may act as an endothelial cell sensor of the matrix microenvironment and thereby regulate endothelial cell production of MMP2 and subsequent endothelial cell invasion and angiogenesis. This concept is supported by TLR4 activation in LECs that was observed in response to Matrigel, which contains a broad array of matrix proteins and constituents. However, further studies are required to ascertain the precise role of endothelial cell TLR4 in the process of matrix sensing.

Activation of TLR4 leads to the downstream activation of the canonical nuclear factor kappa B inflammatory pathway. Indeed, inflammatory cell infiltration is often linked to angiogenesis as a secondary phenomenon because of the release of angiogenic substances by infiltrating inflammatory cells. Thus, a question that emerges from our observations is whether TLR4-induced angiogenesis is driven by direct endothelial cell signaling or rather angiogenesis is a secondary phenomenon that is pursuant to TLR4-induced inflammatory cell infiltration. These directions will be of interest especially in the context of cirrhosis, in which TLR4 function in nearly every liver cell type may be contributing to the fibrosis phenotype.

In summary, the present studies make several new observations that identify innate immune pathways in the process of angiogenesis and its relationship with liver fibrosis. Future studies will be needed to further dissect the precise roles of TLR4 in different hepatic cell populations and their convergent effects on liver fibrosis and its associated changes in vascular structure and integrity.

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