Role of splenic reservoir monocytes in pulmonary vascular monocyte accumulation in experimental hepatopulmonary syndrome

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Key words
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Abstract
Background and Aim: Pulmonary monocyte infiltration plays a significant role in the development of angiogenesis in experimental hepatopulmonary syndrome (HPS) after common bile duct ligation (CBDL). Hepatic monocytes are also increased after CBDL, but the origins remain unclear. Splenic reservoir monocytes have been identified as a major source of monocytes that accumulate in injured tissues. Whether splenic monocytes contribute to monocyte alterations after CBDL is unknown. This study evaluates monocyte distributions and assesses effects of splenectomy on monocyte levels and pulmonary vascular and hepatic abnormalities in experimental HPS.

Methods: Splenectomy was performed in CBDL animals. Monocyte levels in different tissues and circulation were assessed with CD68. Pulmonary alterations of HPS were evaluated with vascular endothelial growth factor-A (VEGF-A) levels, angiogenesis, and alveolar–arterial oxygen gradient (AaPO2). Liver abnormalities were evaluated with fibrosis (Sirius red), bile duct proliferation (CK-19), and enzymatic changes.

Results: Monocyte levels increased in the lung and liver after CBDL and were accompanied by elevated circulating monocyte numbers. Splenectomy significantly decreased monocyte accumulation, VEGF-A levels, and angiogenesis in CBDL animal lung and improved AaPO2 levels. In contrast, hepatic monocyte levels, fibrosis, and functional abnormalities were further exacerbated by spleen removal.

Conclusions: Splenic reservoir monocytes are a major source for lung monocyte accumulation after CBDL, and spleen removal attenuates the development of experimental HPS. Liver monocytes may have different origins, and accumulation is exacerbated after depletion of splenic reservoir monocytes. Tissue specific monocyte alterations, influenced by the spleen reservoir, have a significant impact on pulmonary complications of liver disease.

Introduction
Chronic liver disease leads to altered vascular remodeling and angiogenesis in the hepatic, splanchnic, and systemic circulation.1 Hepatopulmonary syndrome (HPS) is one such vascular complication that develops when liver cirrhosis results in abnormal arterial gas exchange because of alveolar microvascular dilation and remodeling/angiogenesis.2–4 The occurrence of HPS ranges from 5% to 32% in cirrhotic patients evaluated for liver transplantation. The presence of HPS significantly impairs quality of life and increases mortality in affected patients.5 Currently, no effective medical therapies are available because of our limited understanding of pathogenesis.

Experimental HPS induced by common bile duct ligation (CBDL) in the rat is characterized by the development of biliary cirrhosis, portal hypertension, and pulmonary vascular and gas exchange alterations.6,7 A pivotal role of hepatic infiltrating and resident monocytes/macrophages in the pathogenesis of vascular alterations and liver injury has been extensively characterized in a number of models including CBDL.8–9 Our previous work and those by others has demonstrated that pulmonary vascular monocyte infiltration and expression of vascular endothelial growth factors (VEGF-A) is also a key pathogenic event in promoting pulmonary microvascular alterations in experimental HPS.10–12 However, the source of infiltrating monocytes/macrophages in the lung after CBDL has not been defined.

Classically, monocytes derived from progenitor cells in the bone marrow are thought to migrate into the circulation and directly accumulate in tissues in response to acute or chronic injury.13–15 However, recent work has challenged this view and has demonstrated an important role for spleen-released monocytes (or splenic reservoir monocytes) in tissue infiltration, injury, and repair in a number of conditions including myocardial infarction, stroke, atherosclerosis, spinal cord injury, and malignancy.13,16–19 Splenic reservoir monocytes outnumber their circulating counterparts and significantly increase circulating monocyte levels when mobilized.20,21 Whether
the splenic monocyte pool contributes to hepatic and pulmonary monocyte infiltration and HPS after CBDL has not been evaluated.

In the present study, we evaluated monocyte alterations in the circulation, lung, liver, and other organs in experimental HPS and assessed the effect of splenectomy on monocyte levels and pulmonary vascular and hepatic abnormalities.

Methods

Animals. Male Sprague-Dawley rats (250–300 g; Charles River, Wilmington, MA, USA) underwent sham operation (mobilization of the common bile duct without ligation) or CBDL (the common bile duct was doubly ligated and dissected) for 3 weeks as described previously.6,7 Splenectomy was performed in one set of animals at the time of CBDL. Six to eight animals were used in each group. Portal venous pressure (PVP) was measured. Lung, liver, heart, kidney brain, spleen, blood, and plasma were obtained for further analysis. The study was approved by the University of Texas at Houston Health Science Center Animal Welfare Committee and conformed to National Institutes of Health guidelines on the use of laboratory animals.

Arterial blood gas analysis. Arterial blood drawn from the femoral artery as previously described was analyzed on an ABL80 FLEX blood gas analyzer (Radiometer America, Westlake, OH, USA).6 The alveolar–arterial oxygen gradient (AaO2) was calculated as 150 – (PaCO2/0.8) – PaO2.

Histological examination. Five-micrometer sections of 4% paraformaldehyde paraffin-fixed tissue (lung, liver, or spleen) were blocked and incubated with primary antibodies. For immunofluorescent staining of ED1 (CD68, a pan monocyte marker, AbD Serotec, Raleigh, NC, USA) and Factor VIII (FVIII, Cell Marque, Austin, TX, USA), Texas red and fluorescein isothiocyanate-conjugated mouse anti-rat CD68 monoclonal antibody (ED1, a pan monocyte marker, AbD Serotec) and R-phycoerythrin (RPE)-conjugated marker, AbD Serotec) and R-phycoerythrin (RPE)-conjugated mouse anti-rat CD172a monoclonal antibody (CD68+CD172a+) levels. In detail, PBMCs were double-stained by incubating with fluorescein-isothiocyanate-conjugated mouse anti-rat CD68 monoclonal antibody (CD68+CD172a+) and biotinylated secondary antibodies were used. After incubation with streptavidin-biotin-horseradish peroxidase (VECTASTAIN Elite ABC Kit, Vector Laboratories), peroxidase activity was detected with DAB Peroxidase Substrate Kit (Vector Laboratories). Sirius Red (Sigma-Aldrich, St. Louis, MO, USA) staining was performed to evaluate the liver fibrosis. Sections were photographed with a Nikon Eclipse E200 Binocular Microscope (Nikon, Japan). Ten to fifteen fields were analyzed for each section in a blinded manner.

Western blot analysis. Equal amount of proteins from the lung, liver, heart, kidney brain, and spleen were fractionated on Criterion Tris-HCl Gel (4–20%) (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to PVDF membrane (Millipore, Billerica, CA, USA). Incubation with primary antibodies against ED1 was followed by addition of horseradish peroxidase-conjugated secondary antibodies and detection with enhanced chemiluminescence substrate Pico-West luminal reagent (Thermo Scientific Pierce, Waltham, MA, USA). The density of autoradiographic signals was assessed with a ScanMaker i900 scanner (Microtek Lab, Carson, CA, USA) and quantitated with ImageJ software provided by the National Institutes of Health.

Quantitation of pulmonary microvessel densities. The degree of pulmonary angiogenesis was evaluated by quantifying microvessel densities in lung sections immunostained with the endothelial marker Factor VIII as previously described.24 All stained objects were counted in a blinded fashion. Vessels with thick muscular walls or larger than 100 μm in diameter were excluded. Five randomly scanned fields of three sections from each animal were investigated.

RNA extraction and quantitative real-time RT-PCR. Total RNAs from lungs were extracted with Trizol (Invitrogen, Carlsbad, CA, USA) reagent according to the manufacturer’s instructions and treated with RNase-free DNase I (Invitrogen) following the manufacturer’s protocol. cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems Inc., Foster City, CA, USA). Real-time PCR analysis was performed using the StepOnePlus Real-Time PCR System and TaqMan Gene Expression Master Mix (Applied Biosystems Inc.) according to the manufacturer’s recommendations. TaqMan Gene Expression Assay for rat VEGF-A was purchased from Applied Biosystems. Expression levels were normalized to expression of 18S rRNA.

Peripheral blood mononuclear cell preparation and flow cytometry. Whole blood samples were collected from sham and CBDL rats. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll (GE Healthcare Life Sciences, Pittsburgh, PA, USA) density gradient separation method. Flow cytometry (FACS) analysis was performed to measure monocyte (CD68+/CD172a+) levels. In detail, PBMCs were double-stained by incubating with fluorescein-isothiocyanate-conjugated mouse anti-rat CD68 monoclonal antibody (ED1, a pan monocyte marker, AbD Serotec) and R-phycocerythrin (RPE)-conjugated mouse anti-rat CD172a monoclonal antibody (monocyte marker, AbD Serotec) followed by analysis with Gallios flow cytometer (Beckman Coulter, Brea, CA, USA) and FlowJo software (version 9.6.4; Tree Star Inc, Ashland, OR, USA).

Liver functional measurements and circulating TNF-α level assessment. The concentrations of bile acid, bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in plasma were measured respectively using Direct and Total Bilirubin Reagent (GenWay Biotech, Inc, San Diego, CA, USA), Liquid ALT (SGPT) Reagent Set, and Liquid AST (SGOT) Reagent Set (Pointe Scientific, Canton, MI, USA) following manufacturer instructions. The concentrations of TNF-α in plasma were measured using Rat TNF-alpha Quantikine ELISA Kit (R&D Systems, Minneapolis, MN, USA) following manufacturer instructions.

Statistics. Data were analyzed with the Student’s t-test or the analysis of variance with Bonferroni correction for multiple comparisons between groups. Measurements are expressed as means ± SE. Statistical significance was designated as P < 0.05.
Results

**Tissue specific monocyte infiltration and circulating monocyte levels in experimental hepatopulmonary syndrome.** To evaluate monocyte dynamics in experimental HPS, we measured ED1 (CD68, a pan monocyte marker) protein levels in the lung, liver, spleen, and other organs using western blots or immunohistochemistry and assessed the levels of peripheral monocytes (CD68+/CD172a+) by flow cytometry (Fig. 1). Relative to control, monocyte levels in the lung and liver increased within 1 week after CBDL and rose to nearly sixfolds to eightfolds 3 weeks after surgery consistent with previous results. Levels of peripheral monocytes also increased within 1 week after CBDL and further increased at 3 weeks. In contrast, the numbers of splenic monocytes decreased to approximately 30% of control values at 1 week after CBDL, with the reduction persisting after 3 weeks. The levels of monocytes in the heart, kidney, and brain remained unchanged over the 3-week timeframe. These findings demonstrate that CBDL results in specific monocyte recruitment to the lung and liver, which is associated with a reduction in splenic monocytes.

**Effects of splenectomy on pulmonary and hepatic monocyte accumulation.** To determine whether the splenic reservoir pool contributes to pulmonary vascular and liver monocyte accumulation, we performed splenectomy in a set of CBDL animals and evaluated hepatic and pulmonary monocyte levels by western blotting or immunohistochemistry (Fig. 2). Relative to CBDL animals, splenectomized CBDL animals had significantly decreased monocyte accumulation in the lung. However, hepatic monocyte levels increased dramatically in these animals (Fig. 2). These results support that splenectomy in specific monocyte recruitment to the lung and liver, which is associated with a reduction in splenic monocytes.

**Effects of splenectomy on experimental hepatopulmonary syndrome.** To investigate whether the removal of splenic monocyte reservoir influences pulmonary vascular alterations after CBDL, we assessed the levels of pulmonary VEGF-A expression and microvessel density and analyzed arterial blood gasses (Fig. 3). Relative to CBDL controls, pulmonary VEGF mRNA levels and microvessel counts (by Factor VIII staining) were significantly reduced in CBDL animals that underwent splenectomy. These events were associated with improved gas exchange reflected by decreased AaPO2 levels.

**Effects of splenectomy on hepatic alterations and circulating TNF-α levels after common bile duct ligation.** To determine the hepatic consequences of spleen removal after CBDL, we measured portal venous pressure (PVP) and serum levels of aminotransferases, bilirubin, and TNF-α concentration (Table 1) and performed CK-19 and Sirius Red staining in livers (Fig. 4). Relative to CBDL animal without splenectomy, removal of spleen further increased serum levels of ALT and AST. Hepatic Sirius Red staining increased in CBDL animals after spleen removal, indicating enhanced fibrosis. In addition, circulating TNF-α levels were further increased in asplenic CBDL animals (2.36 ± 0.27-fold vs CBDL alone, P < 0.05). However, portal pressure, bilirubin levels, and bile duct proliferation (CK-19 staining) were unchanged over the time frame of the study. These findings suggest that the increase in hepatic monocytes by splenectomy worsens liver injury and fibrosis in contrast to the effects in the lung.

Discussion

In the present study, we have found preferential recruitment of monocyte/macrophages to the liver and lung with a concomitant decline in splenic reservoir monocytes in experimental HPS induced by CBDL. Splenectomy, at the time of CBDL, significantly decreases the recruitment of lung intravascular monocytes and improves the molecular and physiologic features of HPS. In contrast, hepatic monocyte recruitment is significantly increased after splenectomy, and hepatic inflammation and fibrosis are exacerbated. These findings support a central role for the spleen and splenic reservoir monocytes in driving the development of experimental HPS and potentially in mitigating liver injury.

The concept that monocyte infiltration and macrophage activation contribute to organ injury is well recognized and is an important mechanism in liver injury and fibrosis. Our prior studies and those of others also show that microvascular monocyte recruitment in the lung contributes to vascular remodeling and experimental HPS. Recruitment to the lung appears to be driven, in part, by activation of the CX3CL1/CX3CR1 chemokine system. Our finding that monocyte accumulation is minimal in the brain, heart, and kidney after CBDL, despite increased circulating monocyte levels and dysfunction described in these organs, suggests that tissue monocyte accumulation is not a non-specific consequence of inflammation after CBDL. Therefore, dysfunction in the organs other than the liver and the lung after CBDL is likely because of other cell types and mechanistic pathways.

The role of spleen-derived monocytes in modulating tissue injury has been increasingly recognized in experimental models and in humans, including in myocardial infarction, atherosclerosis, stroke, spinal cord injury, and tumor development. In these conditions, the splenic monocyte reservoir egresses from the spleen in response to angiotensin II (AngII)–angiotensin type 1 receptor or chemokine signaling and accumulates into areas of injury and inflammation and infiltration in the abdominal aorta. Inhibiting C-C chemokine receptor 2 expression in splenocytes using siRNA in a mouse model of lung adenocarcinoma reduced monocyte recruitment and tumor progression in the lung. Selective depletion of the splenic reservoir via splenectomy decreases monocyte infiltration in association with attenuated chronic inflammation in these models. Our findings that splenic monocyte levels decrease after CBDL and that splenectomy in CBDL animals prevents lung accumulation of monocytes extend this concept from monocyte alteration of tissue in hepatitis to the lung and the liver, which is associated with a reduction in splenic monocytes.
Monocyte levels in tissues and circulation in experimental hepatopulmonary syndrome. The lung, liver, heart, kidney, brain, and spleen tissues as well as circulating peripheral blood mononuclear cell were obtained from sham, 1 week, and 3 weeks common bile duct ligation (CBDL) animals. Monocyte accumulation in tissues were assessed by the protein levels of ED1 (CD68, a pan monocyte marker) using western blots or immunohistochemistry. Peripheral monocyte levels were assessed by flow cytometry (CD68+/CD172a−, shown by red square). ED1 protein levels were normalized to the levels of glyceraldehyde phosphate dehydrogenase (GAPDH). Increased monocyte infiltration was seen in the lung (a) and liver (b) after CBDL, associated with elevated peripheral monocyte numbers (c). In contrast, splenic monocytes levels decreased after CBDL (d). The levels of monocytes remained unchanged in the heart (e), kidney (f), and brain (g). Values are expressed as means ± SME (n=6–8 animals for each group). *P<0.05 compared with control.
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Figure 2 The effects of spleen removal on pulmonary and hepatic monocyte accumulation. The lung and liver were obtained from common bile duct ligation (CBDL) animals with and without splenectomy. Monocyte levels were assessed by the protein levels of ED1 (CD68, a pan monocyte marker) using western blots or immunohistochemistry. (a) Representative images of immunohistochemical staining for CD68 in the lung and liver; (b) representative immunoblots; and (c) graphical summaries of ED1 protein levels in the lung and liver. Splenectomy significantly decreased monocyte levels in the lung but further increased liver monocyte infiltration. Values are expressed as means ± SE (n = 6–8 animals for each group). *P < 0.05 compared with sham. †P < 0.05 compared with CBDL.

After CBDL, removal of the splenic monocyte reservoir results in distinct consequences within the two organs. In the liver, splenectomy further augmented monocyte/macrophage accumulation and worsened liver fibrosis and dysfunction. These findings suggest that the spleen-derived monocyte subset exhibits anti-fibrotic properties in the liver and that removal of this specialized population exacerbates pro-fibrotic monocyte accumulation and hepatic inflammation. Elevated TNF-α levels have been reported in CBDL animal serum because of liver injury induced endotoxemia and/or bacterial translocation. In parallel with worsened liver injuries, splenectomy also resulted in further increases in circulating TNF-α concentration after CBDL. However, the precise mechanisms through which splenic reservoir monocytes dampen hepatic monocyte infiltration and inflammation remain undefined. Future studies will focus on detailed characterization of the splenic, circulating, and tissue monocyte populations to define the subtypes and mechanisms involved in the distinct lung and liver responses after CBDL.

Despite the emerging role of the spleen as a primary site for monocyte production in tissue injury, alternative mechanisms have been explored in regulating monocyte accumulation and responses at sites of injury. A recent study using mice that express a specific photoconvertible (KiKGR) or cell cycle indicator proteins (Fucci) has reinforced the significance of bone marrow in supplying monocyte accumulation in tumors. This work demonstrated that the bone marrow provides the majority of tumor-infiltrating monocytes and supplies the expansion of splenic monocytes. In addition, it has also been reported that local macrophage proliferation rather than exogenous monocyte infiltration contributes to macrophage increasing in atherosclerosis and adipose tissue inflammation. Therefore, systematic investigations are needed to profile tissue monocyte subsets regarding their origin (spleen vs bone marrow), function (pro- vs anti-inflammation), and relation to tissue macrophages (infiltration vs proliferation) in experimental HPS.

Taken together, the present study establishes the novel concept that tissue-specific monocyte alterations, modulated by splenic reservoir monocytes, influence the end organ complications of liver disease. Specifically, the splenic reservoir is the major source for pulmonary-infiltrating monocytes in experimental HPS, while hepatic monocyte alterations, although influenced by the spleen reservoir, are derived from distinct subsets. Exploring mediators

Figure 3 The effects of spleen removal on pulmonary angiogenesis and gas exchange in experimental hepatopulmonary syndrome. Lung microvessels were stained by Factor VIII (in green) and counted in common bile duct ligation (CBDL) animals with or without splenectomy. (a) Representative immunofluorescent images of Factor VIII; (b) quantitation of Factor VIII-stained microvessels; (c) graphical summaries of lung vascular endothelial growth factor-A (VEGF-A) mRNA levels; and (d) arterial gas exchange was assessed by the alveolar-arterial oxygen gradient (AaPO2). Splenectomy significantly decreased lung microvessel numbers and VEGF-A levels and improved AaPO2 after CBDL. Values are expressed as means ± SE (n = 6–8 animals for each group). *P < 0.05 compared with sham. †P < 0.05 compared with CBDL.

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that drive egress of splenic monocytes and modulate the features of tissue monocyte accumulation in liver disease will provide mechanistic insights into the pathogenesis of HPS and the progression of liver injury.

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