Placental Growth Factor Inhibition Targets Pulmonary Angiogenesis and Represents a Novel Therapy for Hepatopulmonary Syndrome in Mice

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Hepatopulmonary syndrome (HPS) is a severe complication of cirrhosis with increased risk of mortality. Pulmonary microvascular alterations are key features of HPS; but underlying mechanisms are incompletely understood, and studies on HPS are limited to rats. Placental growth factor (PIGF), a proangiogenic molecule that is selectively involved in pathological angiogenesis, may play an important role in HPS development; however, its role has never been investigated. In this study, we validated an HPS model by common bile duct ligation (CBDL) in mice, investigated the kinetic changes in pulmonary angiogenesis and inflammation during HPS development, and provide evidence for a novel therapeutic strategy by targeting pathological angiogenesis. Mice with CBDL developed hypoxemia and intrapulmonary shunting on a background of liver fibrosis. Pulmonary alterations included increased levels of proangiogenic and inflammatory markers, which was confirmed in serum of human HPS patients. Increased PIGF production in HPS mice originated from alveolar type II cells and lung macrophages, as demonstrated by immunofluorescent staining. Dysfunctional vessel formation in CBDL mice was visualized by microscopy on vascular corrosion casts. Both prophylactic and therapeutic anti-PIGF (aPIGF) antibody treatment impeded HPS development, as demonstrated by significantly less intrapulmonary shunting and improved gas exchange. aPIGF treatment decreased endothelial cell dysfunction in vivo and in vitro and was accompanied by reduced pulmonary inflammation. Importantly, aPIGF therapy did not affect liver alterations, supporting aPIGF’s ability to directly target the pulmonary compartment. Conclusion: CBDL in mice induces HPS, which is mediated by PIGF production; aPIGF treatment improves experimental HPS by counteracting pulmonary angiogenesis and might be an attractive therapeutic strategy for human HPS. (HEPATOLOGY 2017; 00:000–000)

Hepatopulmonary syndrome (HPS) refers to a clinical triad: (1) impaired arterial oxygenation, (2) pulmonary microvascular alterations, and (3) occurrence in a setting of liver disease. This complication develops in up to 30% of patients with cirrhosis and, if left untreated, carries a poor prognosis. Despite growing knowledge of the mechanisms involved in HPS development, its pathogenesis...
has not been fully unraveled. No effective medical therapies are available, and liver transplantation remains the only curative option. However, successful transplantation is often hampered by progressive hypoxemia, further highlighting the need for discovering new treatments.

Rat studies have shown that endothelin-1-mediated vasodilation, bacterial translocation, and angiogenesis are key components in HPS pathogenesis. Recent reports have also described involvement of the respiratory epithelial compartment, which needs further investigation. Extensive evidence supports angiogenesis as a crucial mechanism in microvascular proliferation and intrapulmonary shunt formation, which is responsible for gas exchange impairment in HPS. Angiogenesis refers to new blood vessel formation from preexisting vessels, an event that is mediated by angiogenic growth factors. Preclinical studies in common bile duct ligation (CBDL) rats demonstrated that multitargeted antiangiogenic therapies improve experimental HPS. This resulted in an ongoing randomized clinical trial that is studying the multi–tyrosine kinase inhibitor sorafenib as treatment for HPS (ClinicalTrials.gov, NCT02021929). Although multitargeted therapies may be successful, their clinical use is often limited by disabling adverse effects, especially in fragile patients including patients with cirrhosis and complications such as HPS. Thus, for these patients there is still an important unmet medical need.

The development of novel therapies is based on preclinical studies using established animal models covering the pathognomonic features of human disease. At present, CBDL in rats is the only reported animal model for HPS that mimics the pulmonary vascular abnormalities of human HPS. However, in-depth investigation of pathogenic mechanisms relies on the use of transgenic models and antibody-mediated inhibition studies which require a mouse model. As the development of pulmonary angiogenesis during the onset and progression of biliary liver fibrosis in mice has not been outlined yet, we first aimed to validate CBDL in mice as a model for human HPS by focusing on impaired gas exchange and pulmonary vascular alterations during the kinetics of HPS development.

Placental growth factor (PIGF) is a key proangiogenic molecule and a member of the vascular endothelial growth factor (VEGF) family and exclusively regulates angiogenesis in pathological conditions. PIGF specifically binds to VEGF receptor 1 (VEGFR1), its soluble form, and the coreceptors neuropilin 1/2. PIGF stimulates endothelial cell growth, migration, and survival in two ways: (1) indirectly by displacement of VEGF from VEGFR1, thereby triggering VEGF/VEGFR2 signaling pathways and (2) directly by VEGFR1 activation and stimulation of crosstalk between VEGFR1 and VEGFR2. In addition, anti-PIGF (zPIGF) antibodies have been shown to efficiently and selectively inhibit pathological angiogenesis with a favorable safety profile and without the typical side effects seen with other antiangiogenic regimens. Moreover, most angiogenic inhibitors encounter the development of resistance to therapy based on an escape mechanism through “switch on” of compensatory proangiogenic programs. Besides producing a prosurvival effect on endothelial cells, PIGF also attracts VEGFR1-expressing macrophages and bone marrow progenitors, which further stimulate the angiogenic process by additional growth factor release. Unlike other

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antiangiogenic agents, zPIGF therapy has been shown to prevent infiltration with angiocompetent macrophages, which might abolish this antiangiogenic escape.\(^{19,20}\) As such, zPIGF might represent an attractive tool in the therapy for diseases that are mediated by excessive angiogenesis, including HPS.

In the current work, we first validated a mouse model for HPS, evaluated the contribution of PlGF in this model, and then explored its potential as therapeutic target.

### Materials and Methods

#### ANIMALS

Seven-week-old male Swiss mice (Harlan, Brussels, Belgium) were housed in open cages in a temperature-controlled room at 20 °C with a 12 hour dark/12 hour light cycle at the animal facility of the Faculty of Medicine and Health Sciences (Ghent University, Belgium). Animals had free access to water and commercial chow (mouse maintenance chow; Carfil Labofood, Belgium). Mice were acclimatized under controlled conditions for 1 week prior to CBDL or sham surgery, which were performed as described\(^{21}\) (Supporting Information). All mice received care in accordance with the Guide for the Care and Use of Laboratory Animals and the Belgian national guidelines for animal protection. The Ethical Committee of Experimental Animals at the faculty of Medicine and Health Science, Ghent University, Belgium, approved the experiments (ECD 14/67).

#### KINETIC STUDY ON DEVELOPMENT OF HEPATIC FIBROSIS AND PULMONARY ANGIogenesis AND INFLAMMATION

To investigate hepatic and pulmonary angiogenic and inflammatory changes during the development of biliary fibrosis and HPS following CBDL, mice were sacrificed on a weekly basis for 6 consecutive weeks, starting 1 week postsurgery (n = 6-14 animals per group). At sacrifice, serum, bronchoalveolar lavage fluid (BALF), lungs, liver, and spleen were collected for analysis. In a separate experiment, the pulmonary vascular network of sham-operated (n = 3) and CBDL mice with cirrhosis (n = 3) was visualized by scanning electron microscopy (SEM) on vascular corrosion casts.

#### DETERMINATION OF INTRAPULMONARY SHUNT FORMATION

The degree of intrapulmonary shunting using microspheres was assessed by a modification of the methods described by Fallon et al.\(^{17}\) and Miniati et al.\(^{22}\) Sham and CBDL mice were anesthetized with pentobarbital (intraperitoneally, 40 mg/kg, Nembutal; Ceva Santé Animale, Brussels, Belgium), and the inferior caval vein and carotid artery were isolated. Next, \(1 \times 10^6\) fluorescent polystyrene microspheres (6 µm diameter; Phosphorex Inc., Hopkinton, MA) in phosphate-buffered saline were injected into the inferior caval vein. Immediately after injection of the microspheres, the carotid artery was transected and the exsanguinated blood recovered. Fluorescence intensity of the arterial blood was measured on a spectrophotometer (Tecan Safire II, data analysis software Magellan 6; Tecan Trading AG, Switzerland) and corresponds to the degree of intrapulmonary shunting, after correction for collected blood volume.

#### PIGF INHIBITION STUDIES

Anti-PIGF neutralizing monoclonal antibodies (5D11D4; ThromboGenics NV, Leuven, Belgium) were tested for their potential to inhibit pathological angiogenesis and gas exchange impairment in CBDL-induced mice, in a preventive and in a therapeutic setting (n = 4-8 animals per group). Sham and CBDL mice received intraperitoneal antibody administration twice weekly, at a dose of 25 mg/kg (concentration 5.4 mg/mL, volume injected 140-185 µL). A group of matched sham and CBDL mice were injected with mouse immunoglobulin G1 (IgG; ThromboGenics NV) as control at the same dose and times as a PlGF-treated mice (concentration 4.46 mg/mL, volume injected 110-225 µL). The dosing schedule of zPIGF was based on previous pharmacokinetic studies in mice.\(^{20,23}\) In the preventive study, treatment was administered starting at the day of surgery and continued until week 6; in the therapeutic study, the treatment regimen was started at the beginning of postsurgery week 2. Mice were sacrificed 6 weeks postsurgery, and arterial blood, serum, BALF, lungs, liver, and spleen were collected for analysis. In a separate experiment, SEM was performed on lung vascular corrosion casts of CBDL mice that were preventively treated with IgG or zPIGF (n = 6 animals).
FIG. 1

A

B

C

D

E

**FIG. 1**
HUMAN SAMPLES

Serum levels of endoglin (ENG) were assessed by a Luminex magnetic bead assay in blood samples from patients with cirrhosis and HPS (n = 30) and without HPS (n = 30). Additional information regarding laboratory technique and demographic and clinical characteristics of the patients included in the study can be found in the Supporting Information.

STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism software version 6.0 (GraphPad, La Jolla, CA) and SPSS Statistics version 23 (SPSS Inc., Chicago, IL). Data distribution was evaluated with the Shapiro-Wilk test. Normally distributed data were analyzed with the Student t test or analysis of variance. In cases of non-normal distributions, data were analyzed using the Mann-Whitney U or Kruskal-Wallis test. Two-tailed probabilities were calculated, and P values < 0.05 were considered significant.

Additional information on the surgical procedures, arterial blood gas analysis, bronchoalveolar lavage and cytospins, vascular corrosion casting, histology, myeloperoxidase assay, enzyme-linked immunosorbent assay (ELISA), multiplex magnetic bead technology, western blot analysis, cell culture, in vitro experiments, and human samples can be found in the Supporting Information.

Results

VALIDATION OF THE HPS MOUSE MODEL AND KINETICS OF PULMONARY ANGIGENESIS AND INFLAMMATION

Clinicopathological Features of Liver Injury After CBDL

To establish an HPS mouse model, we used CBDL, which is an archetype model for obstructive cholestasis, liver fibrosis, and portal hypertension in rats and mice. CBDL resulted in increased liver and spleen weights starting from week 1 and continuing until week 6 and decreased body weight at week 6 postsurgery compared to sham mice (Supporting Table S2). Liver histology after CBDL showed bile duct proliferation, while no morphological changes in sham controls were observed. Liver fibrosis increased from an F0-1 METAVIR score at week 1 to F4 at week 6 after CBDL (Supporting Fig. S1). No significant increases in protein expression of PI GF, VEGF, or VEGFR1/2 could be demonstrated in hepatic tissue of CBDL mice compared to sham mice (Supporting Fig. S4). CBDL Mice Exhibit Hypoxemia and Intrapulmonary Shunting, in Accordance With HPS

To validate CBDL in mice as an experimental HPS model, gas exchange impairment and the presence of intrapulmonary shunting were assessed. At 6 weeks postsurgery, CBDL mice suffered from significant hypoxemia as demonstrated by arterial blood gas analysis (mean partial pressure of arterial oxygen [PaO2] 63.2 ± 9.2 and 92.2 ± 6.7 mm Hg in CBDL and sham, respectively; P = .007, Fig. 1A). The presence of intrapulmonary shunting was evaluated using intravenous microsphere injections. Because the diameter of the normal mouse pulmonary microvasculature measures <6 μm, most of the microsphere beads injected into the inferior caval vein are trapped in the lung. In HPS, microspheres are able to pass through intrapulmonary vascular dilations and shunts, and the beads can be detected in the arterial blood. Intrapulmonary shunting developed from week 1 after CBDL (P = 0.017 versus sham) and further aggravated until week 6 (P = 0.0045 versus sham) (Fig. 1A). These observations at 6 weeks post-CBDL in conjunction with biliary liver damage and cirrhosis (Supporting Fig. S1) fulfill the clinical criteria for HPS diagnosis and validate this mouse model for HPS.

**Fig. 1.** Validation of CBDL in mice as a model for human HPS. (A) HPS was diagnosed by detection of hypoxemia on arterial blood gas analysis (left graph) and intrapulmonary shunting as evidenced by significantly more fluorescence-labeled microspheres in the arterial blood (right graph). (B) SEM photographs of vascular corrosion casts of sham and CBDL mouse lungs 6 weeks postsurgery. Original magnifications, ×326, ×356, and ×1,420. (C) ENG concentration in sera of patients with cirrhosis and HPS and without HPS as measured by Luminex magnetic bead assay. (D) Quantification of vWF and ENG immunohistochemical staining (mean area ± SE) on sham and CBDL mice lungs at consecutive induction. Original magnification, ×200. (E) Serum concentration of VCAM-1 in CBDL mice as measured by ELISA. Values are represented relative to the mean of the corresponding sham group as fold sham. Data are represented as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.
FIG. 2

A

PIGF (fold sham)

Wk 1  Wk 2  Wk 3  Wk 4  Wk 5  Wk 6

B

PIGF

C

PIGF / ITF-1

D

PIGF / CD68

sVEGFR1 (fold sham)

Wk 1  Wk 2  Wk 3  Wk 4  Wk 5  Wk 6

** P = .06 ** ** ** **

FIG. 2
Architectural changes of the pulmonary vasculature were further investigated by microscopic evaluation of lung vascular casts. Lungs of mice with cirrhosis displayed a chaotically disorganized pulmonary vascular network, marked by tortuosity, scattered lumpy regions, and global distortion of the normally well-organized honeycomb-like capillary network seen in sham mice (Fig. 1B), consistent with previous studies.\(^{(24,25)}\)

**Development of HPS Is Characterized by Pulmonary Angiogenesis**

**EXPERIMENTAL HPS INDUCTION ACTIVATES THE PULMONARY ENDOTHELIUM, WHICH CORRESPONDS TO HUMAN HPS BIOMARKERS**

We and others have previously reported on elevated circulating vascular cellular adhesion molecule 1 (VCAM-1) and von Willebrand factor (vWF) in HPS patients, indicating that these factors may be promising biomarkers for early detection of HPS.\(^{(26,27)}\) To extend these findings, we measured serum levels of ENG, a third marker of endothelial cell activation, in the same patient cohort. In accordance, ENG serum levels had significantly increased in HPS patients compared to non-HPS patients with cirrhosis (mean 6.1 ± 0.6 versus 4.1 ± 0.4 ng/mL, \(P = 0.007\); Fig. 1C). To directly associate these data with pulmonary angiogenesis, we took advantage of our mouse model and analyzed vWF and ENG immunoreactivity in lungs of CBDL and sham mice. As shown by vWF-immunopositive and ENG-immunopositive microvessels, pulmonary angiogenic spots were observed in CBDL mice from week 4 (\(P = 0.05\) versus sham) and progressively increased until week 6 (\(P = 0.0009\) versus sham) (Fig. 1D). VCAM-1 levels were measured in CBDL mice sera because specifically soluble VCAM-1, shed by the endothelium in case of sustained endothelial activation, exerts a proangiogenic function.\(^{(28)}\) Analogous to the human condition, significantly elevated serum levels were observed in CBDL mice, from week 1 (\(P = 0.0048\) versus sham) and continuing until week 6 (\(P = 0.0028\) versus sham) (Fig. 1E).

**PULMONARY ANGIOGENESIS IN EXPERIMENTAL HPS IS MARKED BY AN UP-REGULATION OF PLGF, WHICH IS PRODUCED BY ALVEOLAR TYPE II CELLS AND LUNG MACROPHAGES**

To investigate the involvement of PI GF-mediated signaling in pulmonary angiogenesis, angiogenic markers of the VEGF family were measured at regular intervals following surgery. CBDL led to significant up-regulation of PI GF in the lungs from week 2 (\(P = 0.016\) versus sham) to week 6 (\(P = 0.0019\) versus sham) postinduction (Fig. 2A), while no increase in circulating PI GF could be observed (data not shown) comparable to the human situation.\(^{(26)}\) PI GF colocalized with cluster of differentiation 68 (CD68; monocyte/macrophage marker) and thyroid transcription factor-1 (a nuclear alveolar type II [AT2] cell marker) (Fig. 2B-D); this finding implies that lung macrophages and AT2 cells are the source of PI GF in the lungs of HPS mice. No substantial up-regulation of VEGF or VEGFR1/2 expression in lung tissue of CBDL mice could be demonstrated (Fig. 2A). A significant increase in circulating soluble VEGFR1 was detected from week 1 (\(P = 0.0012\) versus sham) and continued until week 6 (\(P < 0.0001\) versus sham) post-surgery (Fig. 2A).

**Pulmonary Angiogenesis in HPS Development Is Accompanied by Pulmonary Inflammation**

Because PI GF has been linked to angiogenesis-associated inflammation in various pathologies, we determined the time frame in which proinflammatory factors are up-regulated in HPS mice lungs. Increased levels of monocyte chemotactrant protein 1 (MCP-1), which is the main mediator for monocyte attraction to the target site, were observed from week 1 (MCP-1, \(P = 0.001\) versus sham) post-CBDL until endpoint.
This was accompanied by an increase, which was most pronounced during the earlier stages of HPS development, in keratinocyte chemoattractant (KC), which is responsible for neutrophil migration, and interleukin 1β (IL-1β) (Fig. 3A). To investigate leukocyte infiltration, we analyzed pulmonary myeloperoxidase levels, which is an enzyme present in neutrophil granules and monocyte lysosomes, and performed cytological analysis with BALF samples. CBDL caused a significant
increase in pulmonary myeloperoxidase activity (Fig. 3B) and the absolute numbers of neutrophils in BALF compared to sham controls (Fig. 3C). In line with this, levels of neutrophil elastase (NE), which is a protease produced by activated neutrophils that is responsible for breakdown of the alveolar epithelial compartment, were elevated in BALF of CBDL mice in comparison to sham at weeks 5 (P = 0.044) and 6 (P < 0.0001) (Fig. 3D).

**PIGF INHIBITION AMELIORATES CBDL-INDUCED HPS BY COUNTERACTING PULMONARY ANGIOGENESIS**

**Anti-PIGF Antibodies Are Able to Prevent HPS Development in CBDL Mice**

To first assess the role of PIGF in HPS development, CBDL and sham mice were treated with zPIGF antibodies on the day of surgery and continued until week 6. Treatment with zPIGF was well tolerated in sham and CBDL animals, and body weight was similar in mice treated with zPIGF and IgG (Supporting Table S3). Importantly, prophylactic zPIGF treatment was able to prevent pulmonary gas exchange defects following CBDL as observed in IgG-treated CBDL mice (mean PaO₂ 90.5 ± 6.2 versus 63.2 ± 9.2 mm Hg; P = 0.04; Fig. 4A). This was confirmed by lower arterial fluorescence intensity following intravenous microsphere injection as a measure for intrapulmonary shunt formation (P = 0.01; Fig. 4A) and partial normalization of the distorted, densely packed pulmonary microvessels as observed by SEM in zPIGF-injected compared to IgG-injected CBDL mice (Fig. 4B).

**HPS in Mice Can Be Reversed by zPIGF Treatment**

As a next step, we were interested in the therapeutic potential of zPIGF therapy for treating established HPS. Anti-PIGF antibodies were administered starting at the beginning of week 2 post-CBDL, when intrapulmonary shunting is known to be advanced (Fig. 1A). Administration of zPIGF to HPS mice resulted in improved pulmonary gas exchange (mean PaO₂ 90.1 ± 6.5 mm Hg in zPIGF versus 63.4 ± 7.5 mm Hg in IgG-treated mice; P = 0.03), with reduction of intrapulmonary shunting (P = 0.01) (Fig. 5A). The treatment regimen was well tolerated, and no significant effects on body weight were observed (Supporting Table S3).

**PIGF Inhibition Improves HPS Through Pulmonary Endothelial Normalization Without Induction of a Rescue Angiogenic Program**

Based on the obtained results, we hypothesized that zPIGF’s beneficial effect on experimental HPS could be attributed to modulation of pulmonary endothelial dysfunction. Lungs of IgG-treated CBDL mice were marked by scattered vWF-immunopositive and ENG-immunopositive endothelial spots (preventive vWF, P < 0.0001; ENG, P < 0.0001 versus IgG sham, Fig. 4C; therapeutic setting vWF, P = 0.0003; ENG, P = 0.0004 versus IgG sham; Fig. 5B), whereas CBDL mice that received zPIGF showed decreased vWF (P = 0.003 and P = 0.0008 versus IgG CBDL) and ENG (P = 0.0008 and P = 0.01 versus IgG CBDL mice) (Figs. 4C and 5B), which is indicative of reduced endothelial cell activation. This was accompanied by a trend toward lower circulating VCAM-1 in zPIGF-treated compared to IgG-treated CBDL mice (preventive, P = 0.09; therapeutic, P = 0.36; Figs. 4D and 5C).

Because antiangiogenic regimens are known for their potential to induce a rescue angiogenic program, we measured the expression of other proangiogenic member molecules of the VEGF family. Anti-PIGF antibody administration did not induce compensatory expression of VEGF or VEGFR1/2, indicating a negligible induction of angiogenic escape mechanisms (Figs. 4D and 5C).

**PIGF Inhibition Attenuates Pulmonary Inflammation**

Because angiogenesis and inflammation are closely linked, we were interested in whether PIGF activity suppression and the associated endothelial normalization also affected pulmonary inflammation in HPS. Anti-PIGF treatment resulted in a marked reduction in pulmonary MCP-1 expression (preventive, P = 0.06; therapeutic, P = 0.01 versus IgG-treated CBDL; Figs. 4E and 5D), which was the only chemokine that was up-regulated at end-stage fibrosis with HPS. Because MCP-1 mediates the attraction of monocytes/macrophages to the target site, we assessed pulmonary CD68 expression. In sham animals, CD68 levels were minimal, while a significant increase was
FIG. 4
seen in CBDL mice (preventive, sham versus CBDL IgG, $P = 0.0001$; therapeutic, sham versus CBDL IgG, $P = 0.0276$) (Figs. 4F and 5E). Anti-PIGF-treated animals showed reduced CD68 expression compared with untreated CBDL animals (preventive, $P = 0.0028$; therapeutic, $P = 0.05$) (Figs. 4F and 5E), with lower macrophage histological counts (hematoxylin and eosin sections, preventive, $P = 0.0084$ and therapeutic, $P = 0.033$; CD68 sections, preventive, $P = 0.0021$ and therapeutic, $P = 0.24$) (Supporting Fig. S3). In addition, NE levels in BALF were lower (although not significant) in zPIGF-treated CBDL mice compared to IgG-treated animals (preventive, $P = 0.067$; therapeutic, $P = 0.071$) (Supporting Fig. S4).

**Modulation of PIGF Signaling Modifies Endothelial Cell, Macrophage, and AT2 Cell Activation in Response to Lipopolysaccharide In Vitro**

*In vitro* experiments were designed to further explore the behavior of the cells lining the alveolar septum. Lipopolysaccharide (LPS) was selected as the stimulant because bacterial translocation associated with liver disease is seen as the initial trigger for HPS development.

To verify the normalizing effect of zPIGF on the endothelium, MS1 endothelial cells were stimulated with LPS in the presence or absence of zPIGF. Incubation with LPS alone induced secretion of MCP-1, KC, and granulocyte colony-stimulating factor (G-CSF) (all $P < 0.0001$) and VCAM-1 expression ($P = 0.0047$), which was significantly less pronounced during zPIGF cotreatment (Fig. 6A).

In parallel, bone marrow–derived macrophages stimulated with LPS secreted MCP-1, KC, G-CSF (all $P < 0.0001$), and IL-1β ($P = 0.005$). However, addition of zPIGF to cell medium only resulted in less MCP-1 secretion ($P = 0.026$) compared to LPS challenge only (Fig. 6B).

MLE-12 alveolar epithelial cells stimulated with LPS showed elevated MCP-1 ($P < 0.0001$), KC ($P = 0.0003$), and G-CSF ($P = 0.0015$) secretion, along with increased caspase 3/7 activity ($P = 0.0078$), which was inhibited by the addition of zPIGF (MCP-1, $P = 0.0148$; KC, $P = 0.082$; G-CSF, $P = 0.019$; caspase 3/7, $P = 0.023$) (Fig. 6C).

**PIGF INHIBITION DOES NOT AFFECT THE LIVER FOLLOWING CBDL**

We and others have reported on the therapeutic effects of PIGF blockade on liver fibrosis in CCl4-induced mice. Although HPS develops on a background of liver fibrosis, this could only be confirmed following CBDL. To investigate whether zPIGF’s therapeutic effects on HPS and pulmonary alterations are due to reduced liver disease in CBDL mice, we compared the degree of liver fibrosis and hepatic expression of proangiogenic and inflammatory markers in CBDL mice treated with either zPIGF or IgG. CBDL IgG-treated animals showed significantly increased hepatic fibrosis compared to sham controls (preventive, $P = 0.0004$; therapeutic, $P < 0.0001$; Fig. 7A), without any difference after zPIGF treatment (preventive, $P = 0.5$; therapeutic, $P = 0.36$) (Fig. 7A). Although hemodynamic measurements were not performed, histological evaluation of the mesentery showed no effect of PIGF blockade on the splanchic vascular bed (Supporting Fig. S5), and spleen enlargement, a surrogate indicator of portal hypertension, was unaltered following zPIGF treatment (preventive, $0.21 ± 0.030$ versus $0.23 ± 0.043$ g; $P = 0.95$; therapeutic, $0.32 ± 0.034$ versus $0.25 ± 0.030$ g; $P = 0.16$) (Supporting Table S3). MCP-1 and KC levels were markedly elevated in hepatic tissue of CBDL mice, with lower macrophage histological counts (hematoxylin and eosin sections, preventive, $P = 0.0084$ and therapeutic, $P = 0.033$; CD68 sections, preventive, $P = 0.0021$ and therapeutic, $P = 0.24$) (Supporting Fig. S3).

**FIG. 4.** Effects of zPIGF on HPS development, pulmonary angiogenesis, and inflammation in a preventive setting. Sham and CBDL mice were treated with 25 mg/kg zPIGF or IgG twice weekly by intraperitoneal injection starting from surgery until the day of sacrifice at week 6. (A) Left graph: PaO₂ measured on arterial blood gas analysis. Right graph: Fluorescence of arterial blood, representing the degree of intrapulmonary shunting. (B) SEM photographs of vascular corrosion casts of lungs of CBDL mice treated with IgG (left image) or zPIGF (right image). Original magnifications, $× 549$ and $× 526$. (C) Quantification of pulmonary vWF and ENG expression (mean area) by immunohistochemistry and representative images of ENG immunohistochemical staining on lungs of CBDL mice treated with IgG (left image) or zPIGF (right image). Original magnification, $× 200$. (D) Serum levels of VCAM-1 and pulmonary expressions of PIGF, VEGF, and VEGFRI/2 as measured by ELISA. Values are represented relative to the mean of sham IgG-treated mice as fold sham IgG. (E) Pulmonary expression of MCP-1 as measured by multiplex magnetic bead technology. Values are represented relative to the mean of sham IgG-treated mice as fold sham IgG. Data are represented as means ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

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FIG. 5. Effects of sPlGF on HPS, pulmonary angiogenesis, and inflammation in a therapeutic setting. Sham and CBDL mice were treated with 25 mg/kg sPlGF or IgG twice weekly by intraperitoneal injection starting at the beginning of week 2 postsurgery until the day of sacrifice at week 6. (A) Left graph: PaO2 measured on arterial blood gas analysis. Right graph: Fluorescence intensity of arterial blood, corresponding to the degree of intrapulmonary shunting. (B) Quantification of pulmonary vWF and ENG expressions (mean area) by immunohistochemistry and representative images of ENG immunohistochemical staining on lungs of CBDL mice treated with IgG (left image) or sPlGF (right image). Original magnification, ×200. (C) Serum levels of VCAM-1 and pulmonary expressions of PIGF, VEGF, and VEGFR1/2 as measured by ELISA. Values are represented relative to the mean of sham IgG-treated mice as fold sham IgG. (D) Pulmonary expression of MCP-1 as measured by multiplex magnetic bead technology. Values are represented relative to the mean of sham IgG-treated mice as fold sham IgG. (E) Pulmonary protein expression (normalized volume) of CD68 determined by western blot analysis. Values are represented relative to the mean of sham IgG-treated mice as fold sham IgG. Data are represented as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001.
FIG. 6. Effects of αPlGF on endothelial cell, macrophage, and AT2 cell response to LPS challenge in vitro. MS1 endothelial cells, bone marrow–derived macrophages, and MLE-12 lung epithelial cells were stimulated with LPS to mimic bacterial translocation associated with liver disease. (A) VCAM-1 expression (ELISA) and MCP-1, KC, and G-CSF levels (multiplex magnetic bead technology) in supernatant from unstimulated and LPS–stimulated MS1 cells with or without αPlGF. (B) MCP-1, KC, G-CSF, and IL-1β levels (multiplex bead–based technology) in supernatant from unstimulated and LPS–stimulated bone marrow–derived macrophages with or without αPlGF. (C) MCP-1, KC, and G-CSF levels (multiplex bead–based technology) in supernatant and caspase 3/7 activity in unstimulated and LPS–stimulated MLE-12 cells with or without αPlGF. Data are represented as means ± SE. *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviations: BMDM, bone marrow–derived macrophage; NS, not significant.
FIG. 7
compared to sham mice and remained unaffected after zPlGF treatment (MCP-1 preventive, \( P = 0.38 \); therapeutic, \( P = 0.61 \); KC preventive, \( P = 0.44 \); therapeutic, \( P = 0.38 \)) (Fig. 7C,E). Irrespective of the treatment regimen, neither PlGF, VEGF, nor VEGFR1/2 expressions were up-regulated in CBDL mice livers (Fig. 7B,D).

**Discussion**

Multiple lines of evidence support that pulmonary angiogenesis serves as a principal event in experimental HPS development.\(^8,12,32\) However, reports are limited to observations in rats, and the pathogenesis of pulmonary microvascular alterations in HPS remains poorly understood, which contributes to the lack of effective medical therapies. In this article, we describe the establishment of an HPS mouse model, the role of PlGF in HPS development, and the efficacy of zPlGF treatment for HPS in this model.

Several animal models of liver disease have been evaluated for their potential to mimic HPS. So far, only CBDL in rats has recapitulated human HPS.\(^12\) Our first aim was to establish an HPS mouse model in order to expand the possibility of future research on HPS pathogenesis and development of new therapeutic agents. We have documented that CBDL in mice, as in rats, induces HPS. CBDL mice develop intrapulmonary shunting and become hypoxic on a background of cholestatic liver disease, which fulfills the three diagnostic criteria of HPS.\(^1\) To our knowledge, only one study has reported on CBDL in mice as a potential model for HPS, which, however, failed to demonstrate intrapulmonary shunts due to methodological issues.\(^33\) Our findings are in line with knowledge from human patients\(^1,34\) and prior work in rats,\(^8,12,17\) in which it was shown that shunts start to develop early after CBDL induction and do not require advanced fibrosis.

We and others have shown that endothelial dysfunction markers in serum might predict HPS in patients with cirrhosis.\(^26,27\) Our current observation that circulating ENG levels, similar to vWF and VCAM-1, are increased in this same cohort of HPS patients further adds to the concept of enhanced angiogenesis as a pivotal contributor to HPS development. To verify if these specific factors are of actual importance in HPS pathogenesis, their role was assessed in the established HPS mouse model. Pulmonary endothelium of HPS mice specifically expressed vWF and ENG as scattered angiogenic spots and secreted VCAM-1. These events become aggravated over the course of HPS development and are indicative of progressing endothelial dysfunction. The similarities between experimentally induced and human HPS hereby confirm that the CBDL mouse model qualifies as a valuable model to further investigate pathological angiogenesis in the pathogenesis of HPS and will be valuable to test novel treatments.

Although intrapulmonary vasodilation, angiogenesis, and inflammation have been identified to play a key role in HPS pathogenesis, clinical studies targeting these pathways in the past were rather disappointing.\(^34\) At present, sorafenib is being tested as a therapeutic strategy for HPS, but important questions arise with respect to its safety because multitargeted therapies are notorious for their associated toxicities. In this regard, we hypothesized that PlGF, a proangiogenic molecule with restricted activity in pathological conditions, could serve as a potential target for HPS. We have shown that PlGF is up-regulated in CBDL mouse lungs early during HPS development and that PlGF is locally produced by intrapulmonary macrophages and AT2 cells. Our study presents evidence...
concerning the capacity of zPIGF therapy to prevent and reverse experimental HPS in mice with improved gas exchange, decreased intrapulmonary shunting, and attenuation of endothelial activation and pulmonary angiogenesis. Down-regulation of pulmonary vWF and ENG, partial normalization of the three-dimensional structure of the pulmonary vascular network, and amelioration of HPS clinical signs following zPIGF administration further support a direct role for PIGF in HPS and associated pulmonary angiogenesis.

The therapeutic potential of PIGF blockade has been a subject of study in different pathologies, most of which are related to ophthalmology and oncology. Anti-PIGF antibodies have been shown to selectively inhibit pathological angiogenesis, without affecting healthy vessels and thereby causing minimal side effects, and have been demonstrated to be safe and well-tolerated in human healthy volunteers and onologic patients. Previous studies have revealed that PIGF is dispensable for physiological vessel maintenance in healthy mice while contributing to the angiogenic switch in case of pathology. Importantly, zPIGF treatment did not alter expression of VEGF family members in the present study, indicating minimal induction of angiogenic escape.

*In vitro* experiments further support the concept of zPIGF’s normalizing effect on the endothelium as the main underlying mechanism of HPS improvement. This concept was demonstrated by a reduction of VCAM-1 and proinflammatory signature of endothelial cells when subjected to LPS stimulation; these findings are consistent with our *in vivo* observations. Our results further indicated an additional effect on macrophage MCP-1 secretion, which was reduced upon PIGF inhibition. Indeed, zPIGF treatment *in vivo* resulted in decreased expression of proinflammatory markers and CD68 levels. This is a valuable finding because bacterial translocation with subsequent pulmonary inflammation is assumed to be the main driver of HPS onset and might contribute to antiangiogenic escape. Later stages in HPS development in our study were characterized by pulmonary neutrophil infiltration and elevated levels of NE in BALF. Other studies have described a role for elastase and PIGF in inducing AT2 cell death during pulmonary emphysema, and a recent report demonstrated AT2 cell dysfunction after CBDL in rats. In line with this, we have shown that zPIGF attenuates the AT2 cell proinflammatory and caspase 3/7 response to LPS *in vitro*. However, targeting the AT2 cell itself as a novel therapeutic option in HPS warrants further investigation.

Finally, we and others have reported on the potential of PIGF as a therapeutic target in chronic liver disease using the CCl4 model. Based on these results, one could postulate that the beneficial effects of zPIGF on HPS originate from a favorable primary effect on the liver. However, PIGF was not up-regulated in liver tissue of CBDL mice, and no differences in hepatic fibrosis, spleen weight, and inflammatory markers could be demonstrated between zPIGF-treated and IgG-treated CBDL mice. This finding supports zPIGF’s capability to limit HPS through a direct effect on the pulmonary compartment. Even though CCl4 and CBDL are two highly reliable models sharing a common outcome (liver fibrosis), their pathogenic mechanisms are far from comparable. CCl4 leads to centrilobular hepatocyte death and pericentral fibrosis, whereas CBDL results in obstructive cholestasis and bile duct proliferation with portal inflammation and periportal fibrosis. These dissimilarities might explain the differences in hepatic PIGF expression between different animal models and explain why HPS only develops in CBDL-induced mice.

In conclusion, we validated a mouse model for HPS and identified PIGF as an important contributor to pulmonary alterations in HPS pathogenesis. Our data provide evidence for zPIGF antibodies as a therapeutic strategy against HPS, a life-threatening disorder for which no effective medical treatment is available at this time.

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Author names in bold designate shared co-first authorship.

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