# ROLES OF LIVER SINUSOIDAL ENDOTHELIAL CELLS IN LIVER HOMEOSTASIS AND DISEASE

EDITED BY: Patricia Lalor, Leo A. van Grunsven and Thomas Huser
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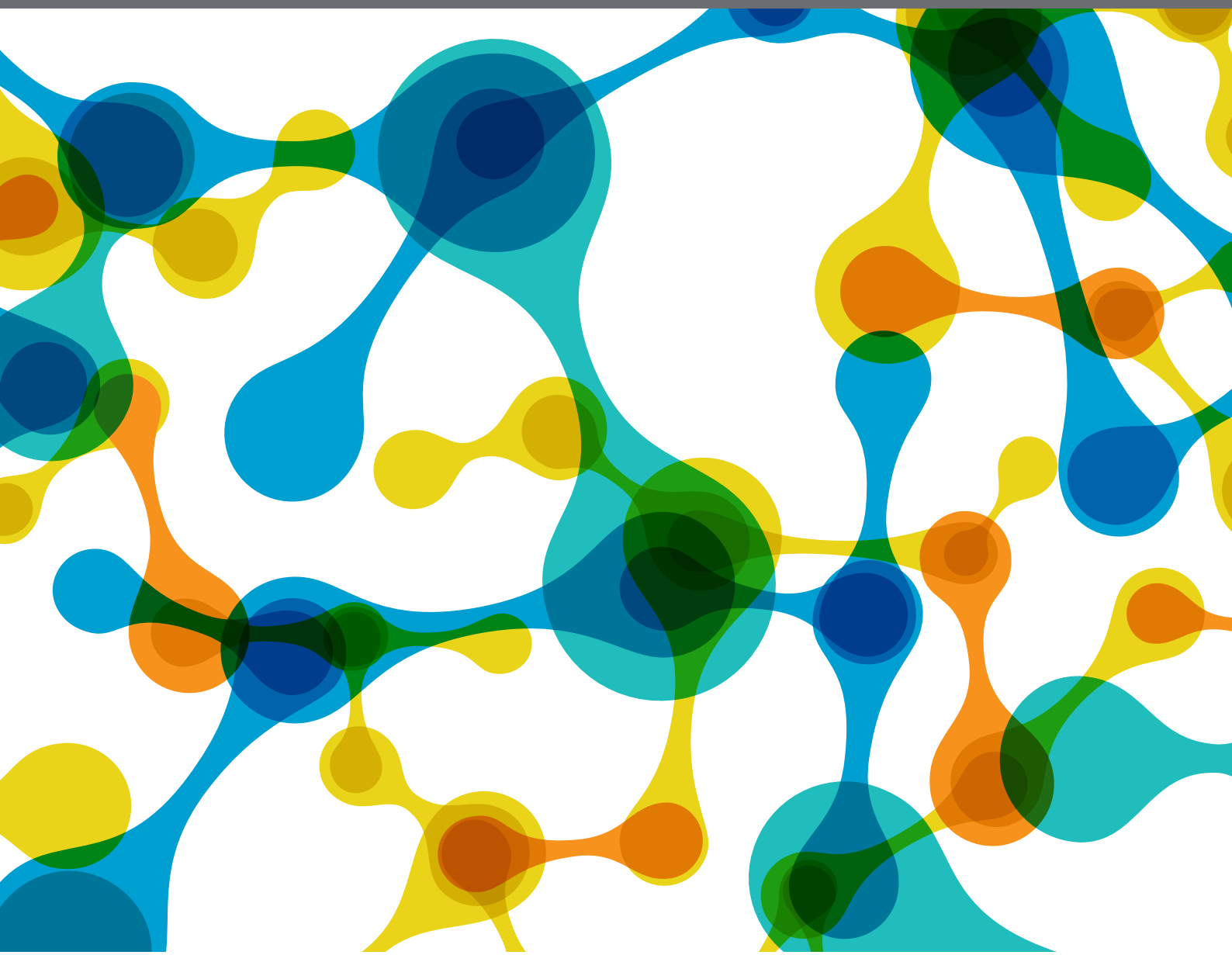

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# ROLES OF LIVER SINUSOIDAL ENDOTHELIAL CELLS IN LIVER HOMEOSTASIS AND DISEASE

Topic Editors:
**Patricia Lalor,** University of Birmingham, United Kingdom
**Leo A. van Grunsven,** Vrije University Brussel, Belgium
**Thomas Huser,** Bielefeld University, Germany

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EDITORIAL
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# Editorial: Roles of Liver Sinusoidal Endothelial Cells in Liver Homeostasis and Disease

*Patricia F. Lalor[1]*, Thomas Huser[2] and Leo A. van Grunsven[3]*

*[1] Centre for Liver and Gastroenterology Research and National Institute for Health Research (NIHR) Birmingham Biomedical Research Centre, Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom, [2] Biomolecular Photonics Research Group, Faculty of Physics, Bielefeld University, Bielefeld, Germany, [3] Liver Cell Biology Research Group, Vrije Universiteit Brussel, Brussel, Belgium*

**Keywords: sinusoidal endothelial cell, liver, chronic liver damage, scavenger receptor, super-resolution imaging, gene expression**

## Editorial on the Research Topic

## Roles of Liver Sinusoidal Endothelial Cells in Liver Homeostasis and Disease

As a consequence of rising global rates of metabolic disease and alcohol-related injury, the research community has a heightened interest in understanding the mechanisms which underlie hepatic inflammation and fibrogenesis. Specific immune cell populations and hepatic stellate cells have obvious significance in this regard, but one underestimated cell population deserves greater prominence. Hepatic sinusoidal endothelial cells are vital to metabolic and functional homeostasis, and changes in their phenotype as a consequence of injury or aging have an impact on disease pathophysiology. In this article series our authors have highlighted both novel mechanistic approaches, and novel features of liver sinusoidal endothelial cells (LSEC) which have great potential for preclinical development.

Bhandari et al. review the phagocytic and clearance roles of LSEC to consider their function as part of the hepatic reticuloendothelial system. Scavenger functions protect liver parenchyma from exposure to noxious substances. Hence scavenger receptors contribute to clearance of viruses, macromolecules, and nanosized pharmaceuticals and also contribute to immune defense by supporting immune cell recruitment. In addition to their consideration of key receptors involved in clearance, the authors also comment on anatomical features of LSEC such as their fenestrations, large surface area and abundant endocytic machinery which support the fast and efficient clearance roles of these cells. There is also consideration of the evolutionary conservation of scavenger endothelial functions to emphasize the conserved importance of the clearance capacities of such cells in vertebrate species. In another contribution, Pandey et al. provide more detail on the structure of hepatic sinusoidal endothelial cells and revisit key scavenger receptors expressed by LSEC to highlight the importance of the clearance capacity of these cells. Here, the authors highlight the diversity of possible ligands cleared by LSEC. They also consider both homeostatic clearance of endogenous lipids and degradation products, and the uptake of lipopolysaccharides (LPS), pathogen associated molecules and oxidized lipids in a diseased context. Changes in receptor expression profiles which occur in chronic disease or cancer in both rodents and humans are also reviewed. Furthermore, they also provide an interesting perspective on LSEC adhesion molecules involved in the regulation of liver inflammation and cell recruitment from the bloodstream, which are important mechanisms for recruiting progenitor cells in the context of disease. Tripathi et al. consider the angiogenic role of circulating bone marrow-derived endothelial progenitor cells in cirrhosis. They show that CD34+VEGFR2+ cells can be detected in human blood and are increased

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***Edited by:***
*Stephen J. Pandol, Cedars Sinai Medical Center, United States*

***Reviewed by:***
*Edward N. Harris, University of Nebraska System, United States*

****Correspondence:***
*Patricia F. Lalor*
*p.f.lalor@bham.ac.uk*

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in cirrhosis. These cells express endothelial-like characteristics when cultured *in vitro* and infiltrate the liver after intravenous administration in rats. There appears to be a change in phenotype of these cells in the context of cirrhosis such that cells from cirrhotic patients seem to enhance rodent fibrotic injury and portal hypertension when compared to cells from healthy individuals. This suggests not only that dedifferentiation of endothelial precursor cells occurs in cirrhosis, but also that normal repair mechanisms which maintain hepatic vascular homeostasis in health may be compromised in chronic injury. Thus, diseased endothelial precursors and local de-differentiated LSEC contribute to fibrogenesis in disease.

Wilkinson et al. turn their attention specifically to LSEC in the context of cancer and chronic disease. Here, they explain the mechanistic basis by which capillarization of LSEC contributes to portal hypertension, altered thrombogenesis, chronic inflammation and perpetuation of fibrogenesis. By focusing on the influence of the microenvironment around LSEC with respect to their function, the authors consider underexplored metabolic and angiogenic aspects of LSEC function, alongside their role in secondary metastasis to highlight novel approaches for cancer therapies. Candidate molecules such as VAP-1 and SCARF-1, and novel approaches using targeted nanoparticles and miRNAs are considered as potential therapeutic tools to manage the increasing burden of liver cancer. In another contribution, a mechanistic explanation for maintenance of LSEC phenotype is provided by Koch et al., who used mice with constitutive activation of β-catenin in endothelial cells. They confirm that a basal low level of *Wnt* signaling is crucial for maintenance of mature LSEC phenotype. Gain of function overexpression in LSEC led to a dedifferentiation response and change in lipoprotein transport accompanied by a loss of prototypic LSEC gene expression. This led to adoption of a phenotype similar to vascular endothelial cells in the blood brain barrier where *Wnt* signaling is normally confined. Whilst not all features of capillarization were recreated by this approach, the study provides insight into key angiocrine signatures which impact endothelial phenotype and lipid metabolism. Important endothelial specific gene signatures are also described in a study by Verhulst et al.. The authors harness the power of single cell sequencing datasets to provide a detailed and important bioinformatic analysis of gene expression in healthy and diseased rodent livers and human samples. This allowed identification of key genes which are differentially expressed in diseased human cells and were linked to fibrogenesis and migration. However, cardinal functions such as scavenging appear conserved even in disease since gene signatures for scavenger receptors and viral coreceptors were enriched in both diseased and healthy samples. Acute injury had a profound effect on murine LSEC phenotype with an upregulation of reparatory and inflammatory signaling which persisted for some time. Importantly a comparison of the gene signatures in human and murine LSEC revealed key genes that can differentiate healthy LSEC from diseased cells in both species.

Much of the available evidence on LSEC function and anatomy has historically been derived from rodent studies, so the human study by Kong et al. is significant. The authors address the challenges of imaging anatomical features and LSEC in human liver specimens by providing beautiful evidence highlighting the potential of different advanced microscopy applications. Mesoscale approaches such as lightsheet microscopy and optical projection tomography provide insights into whole tissue, and gross vascular architectural change in disease. Innovative biophysical imaging tools such as coherent Raman and confocal optical microscopy add subcellular detail in a label-free, minimally processed manner thereby preserving the integrity of valuable biopsy specimens. Here, the potential to assess early fibrogenesis and quantify steatosis is particularly impressive. Finally, the power of super-resolution imaging is demonstrated with structured illumination microscopic images of fenestrations in human LSEC in culture. The fenestrations in LSEC are revisited in the review from Szafranska et al., who consider the impact of endogenous agents and pharmacological compounds on liver endothelial cell porosity. This is important as the pharmacokinetics and clearance of molecules by the liver are impacted by transport across the LSEC. Mechanistic regulation of fenestration size and number is also explained, and the authors consider how extending our knowledge of these processes may facilitate development of tools to restore porosity in aging or chronic disease. Macromolecule clearance is also the focus of an article by James et al. who highlight the underappreciated role of LSEC in immune complex clearance. Here, key scavenger receptors again feature but specifically in regard to their roles in the uptake and recycling of therapeutic antibody. This is relevant for intelligent design of new biological drugs. Strategies to maximize efficacy and minimize antibody clearance, and the impact of disease or aging on pharmacokinetics are described. Consideration is also given to how LSEC scavenger receptor mediated clearance may explain some of the off-target side effects of antibody-based therapies.

In combination, the studies presented within this Research Topic demonstrate the diverse and often underexplored functions of the sinusoidal endothelium and provide a great primer for those striving to learn more about these fascinating cells.

## AUTHOR CONTRIBUTIONS

All authors contributed to the drafting and editing of this document and approved the final version.

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# Cirrhotic Endothelial Progenitor Cells Enhance Liver Angiogenesis and Fibrosis and Aggravate Portal Hypertension in Bile Duct-Ligated Cirrhotic Rats

*Dinesh Mani Tripathi[1†], Mohsin Hassan[1†], Hamda Siddiqui[1], Impreet Kaur[1], Preety Rawal[2], Chaggan Bihari[3], Savneet Kaur[1*] and Shiv K. Sarin[1,4*]*

[1] Department of Molecular and Cellular Medicine, Institute of Liver and Biliary Sciences, New Delhi, India, [2] School of Biotechnology, Gautam Buddha University, Greater Noida, India, [3] Department of Pathology, Institute of Liver and Biliary Sciences, New Delhi, India, [4] Department of Hepatology, Institute of Liver and Biliary Sciences, New Delhi, India

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**Edited by:**
Leo A. van Grunsven,
Vrije University Brussel, Belgium

**Reviewed by:**
Jordi Gracia-Sancho,
August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Spain
Barbara Ruszkowska-Ciastek,
Nicolaus Copernicus University in Toruń, Poland

***Correspondence:**
Savneet Kaur
savykaur@gmail.com
Shiv K. Sarin
shivsarin@gmail.com

[†]These authors share first authorship

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**Background:** Circulating cirrhotic endothelial progenitor cells (EPC) interact with both liver sinusoidal endothelial cells (LSEC) and hepatic stellate cells (HSC) and promote angiogenesis *in vitro*. This study evaluated the effect of cirrhotic and control EPCs on hepatic angiogenesis, microcirculation, and fibrosis *in vivo* in rat models of cirrhosis.

**Methodology:** Animal models of cirrhosis were prepared by bile duct ligation (BDL). Circulating EPCs isolated from healthy human and cirrhotic blood were characterized by flow cytometry, cultured and administered through the tail vein in BDL rats after 2 weeks of ligation. The cells were given thrice a week for 2 weeks. The untreated group of BDL rats received only saline. Fibrosis was evaluated by Masson's trichrome staining. Dedifferentiated LSECs were identified by the expression of CD31, and activated HSCs were marked as alpha-SMA-positive cells and were studied by immunohistochemistry and western blotting in saline-, healthy EPC-, and cirrhotic EPC-treated rats. *In vivo*, hepatic and systemic hemodynamic parameters were evaluated. Liver functions were evaluated.

**Results:** In comparison to controls, BDL rats revealed an increase of fibrosis and angiogenesis. Among the treated rats, cirrhotic EPC-treated rats had increased fibrosis grade as compared to healthy EPC-treated and saline-treated rats. There was an increase of both fibrosis and angiogenesis markers, alpha-SMA and CD31 in cirrhotic EPC-treated rats as compared to healthy EPC-treated and saline-treated rats in immunohistochemistry and western blot studies. Cirrhotic EPC-treated BDL rats had high portal pressure and portal blood flow with significantly elevated hepatic vascular resistance in comparison with healthy EPC- and saline-treated BDL animals, without significant differences in mean arterial pressure. Cirrhotic EPC-treated BDL rats also showed a substantial increase in the hepatic expression of angiogenic receptors, VEGFR2 and CXCR4 in comparison with saline-treated rats.

**Conclusion:** The study suggests that transplantation of cirrhotic EPCs enhances LSEC differentiation and angiogenesis, activates HSCs and worsens fibrosis, thus resulting in hepatic hemodynamic derangements in BDL-induced cirrhosis.

**Keywords: endothelial progenitor cells, fibrosis, angiogenesis, portal hypertension, cell transplant, vascular endothelial growth factor**

## INTRODUCTION

Angiogenesis is the process of formation of new blood vessels from pre-existing vasculature and mature endothelial cells. In liver, physiological angiogenesis occurs during regeneration, and pathological angiogenesis takes place during progression of fibrosis to cirrhosis and during tumorigenesis (Kaur and Anita, 2013; Gracia-Sancho et al., 2019). During both physiological and pathological angiogenesis, cellular cross-talk among several liver cell types such as sinusoidal endothelial cells (LSECs), hepatic stellate cells (HSCs), and hepatocytes orchestrates the angiogenic response in liver. Along with LSECs, bone marrow (BM)-derived endothelial progenitor cells (EPCs) are now well-reported to contribute toward post-natal vasculogenesis/angiogenesis (Asahara et al., 1997; Shi et al., 1998; Marrone et al., 2016). In response to tissue ischemia or traumatic injury, BM-derived EPCs are mobilized into the peripheral blood, migrate to sites of injured endothelium, and henceforth participate into endothelial differentiation and repair (Takahashi et al., 1999; Gill et al., 2001; Kawamoto et al., 2001; Balaji et al., 2013).

Endogenously, these cells express mixed markers present on hematopoietic stem cells and mature endothelial cells such as Vegfr2 and CD34, but do not express CD45 (Kaur and Bajwa, 2014). In culture, they can be grown as early EPCs (<14 days) or late EPCs (>14 days). Importantly, cultured EPCs express endothelial markers including vWF and eNOS (Hirschi et al., 2008). Previous studies have demonstrated that an intraperitoneal administration of EPCs in animal models of dimethylnitrosamine (DMN)- and carbon tetrachloride ($CCl_4$)-induced liver injuries promotes liver regeneration and inhibits progression of liver fibrosis (Fadini et al., 2012; Nakamura et al., 2012). In comparison with the untreated animals, animals receiving EPC therapy are shown to have enhanced expression of regeneration markers, hepatocyte growth factor (HGF), tumor growth factor alpha (TGF-α), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF) and a decreased expression of fibrotic markers, alpha smooth muscle actin (α-SMA), caveolin, and endothelin-1 (Nakamura et al., 2012). The EPC-treated animals also exhibit improvements in liver function parameters including transaminases, total bilirubin, total protein, and albumin (Nakamura et al., 2007, 2012). EPC treatment in CCl4 rats has also been associated with a reduction in portal venous pressure, an increase in portal blood flow, and also an upregulated expression of endothelial nitric oxide synthase (eNOS) and VEGF (Nakamura et al., 2007).

In our previous study, we have demonstrated that in comparison with healthy human subjects, the percentage and proliferation of circulating EPCs are markedly increased in patients with cirrhosis. In these patients, cirrhotic EPCs interact, stimulate the LSECs, and enhance *in vitro* angiogenesis (Sakamoto et al., 2013). In another study, we have reported that BM-EPCs transverse to the liver during CCl4-induced liver injury. We have also shown through *in vitro* studies that EPCs activate HSCs and possibly contribute to *in vivo* fibrosis (Kaur et al., 2012). In this study, we sought to investigate the effect of cirrhotic EPCs on the phenotype and functions of LSECs and HSCs *in vivo* in bile duct models (BDL) of liver fibrosis, that most closely resemble end-stage human liver cirrhosis in many aspects.

## MATERIALS AND METHODS

### Development of Experimental Animal Models of Cirrhosis by Ligation of Common Bile Duct (BDL)

The study was carried out in male Sprague-Dawley rats. All procedures were approved by the Institutional Animal Ethics Committee (IAEC) of the Institute of Liver and Biliary Sciences New Delhi, India, and experiments were conducted in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), New Delhi, India, after approval of IAEC.

Seven-week-old male Sprague-Dawley rats weighing about 200–250 g were taken for the study. Rats were housed at a controlled temperature of 24°C under a 12-h light–dark cycle and were fed standard laboratory chow and water. The surgical procedure for BDL was done under sterile conditions as described elsewhere (Garg et al., 2017). Briefly, animals were anaesthetized with ketamine hydrochloride (100 mg/kg; Neon Laboratories Limited, India) plus midazolam (5 mg/kg; Neon Laboratories Limited, India) intraperitoneally. A mid-line incision was made, and the common bile duct was isolated. On the proximal and distal side of the common bile duct, two ligatures (using silk thread 5-0) were made. The first ligature was made below the junction of hepatic duct and the second above the entry of the pancreatic duct, and a cut was made in between the two ligatures with a fine scissor. All the animals were put for the postoperative care according to the institutional animal facility standard operating procedure. Two weeks after bile duct ligation, the rats were divided into three groups: saline-treated BDL, control EPC-treated BDL, and cirrhotic EPC-treated BDL ($N = 8$ each).

### EPC Culture and Characterization

Circulating EPCs in the peripheral blood were quantified in healthy human subjects and cirrhotic patients ($N = 8$ each) by fluorescent-activated cell sorting (FACS). The characteristics

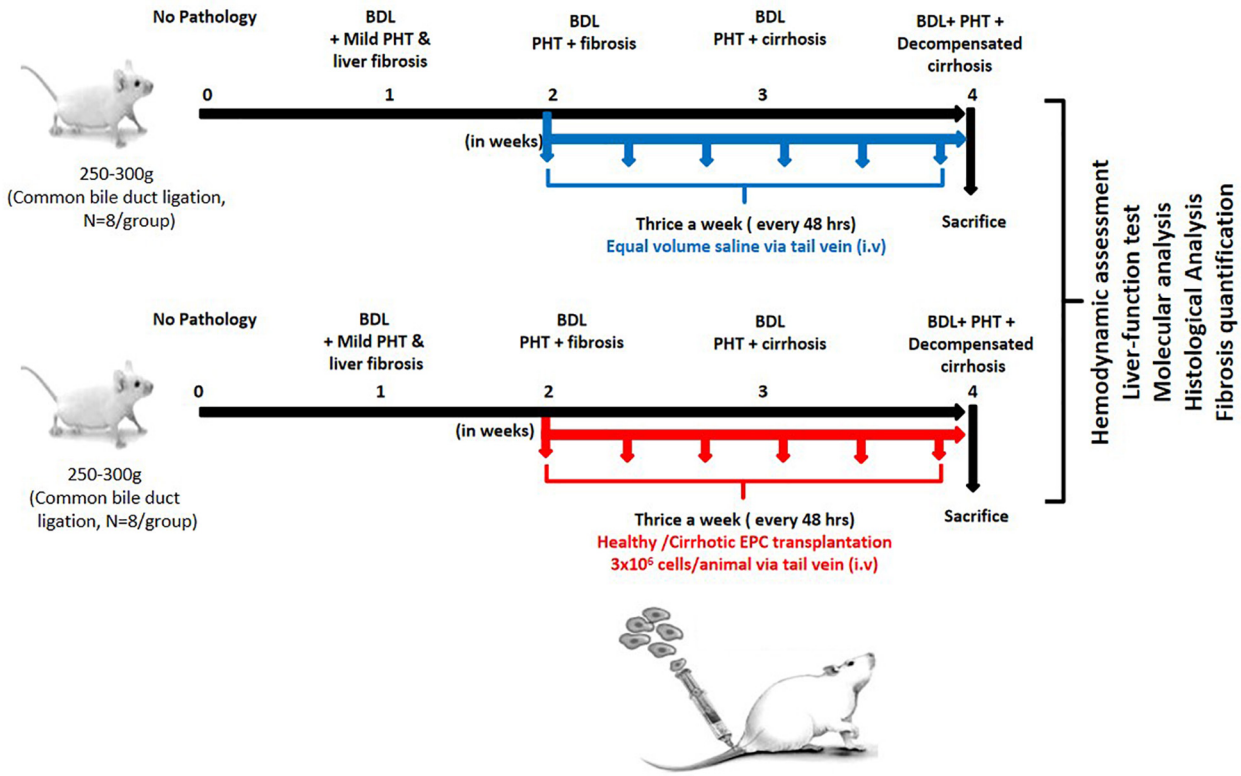


**FIGURE 1 |** Work plan for *in vitro* and *in vivo* studies. In the *in vitro* studies, human EPCs were isolated and cultured from healthy controls ($N$ = 10) and cirrhotic patients ($N$ = 10). In the *in vivo* studies, the cultured EPCs from healthy controls and patients were transplanted into bile duct-ligated rats ($N$ = 8 each) via the tail veil. Saline-treated rats served as the control group ($N$ = 8).

of the cirrhotic patients are given in **Supplementary Table S1**. A total of 2–3 ml of whole blood was used for the isolation of peripheral blood mononuclear cells (PBMCs) by Ficoll method using density centrifugation (Histopaque 1077; Sigma-Aldrich, United States). After RBC lysis, using 1× RBC lysis buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.1 mM EDTA) for 10 min at room temperature, an equal amount of 1× PBS was added. The samples were then centrifuged at 300 × *g* at room temperature. The resulting cell pellet was washed and re-suspended in the appropriate FACS buffer (PBS, 2 mM EDTA, 2% FBS) for further cell surface staining. About 3–4 × $10^6$ cells were stained with the antibodies, anti-human FITC-CD34 (1:100), and anti-human APC-Vegfr2/Flk-1 (1:100) in PBS for 30 min at 4°C (**Supplementary Table S2**) (Kaur and Bajwa, 2014). The cells were then fixed with 4% PFA in PBS and analyzed by BD FACS Aria III (BD Biosciences and DIVA software). A minimum of 100,000 events were acquired for each sample. To nullify non-specific binding, CD34 and Vegfr2 antibodies (Santa Cruz Biotechnology) without any flourophores were used as negative controls).

For culture assays, circulating EPCs were further isolated and expanded *ex vivo* from patients with cirrhosis irrespective of the etiology ($N$ = 10) and healthy controls ($N$ = 10) as previously described (Sakamoto et al., 2013). Briefly, PBMCs were isolated from a 12–15 ml blood sample by density centrifugation (Histopaque 1077, Sigma-Aldrich, United States). After washing with PBS and RBC lysis, PBMCs (1 × $10^6$ cells/$cm^2$) were seeded on fibronectin-coated 6-well plates (Nunc) in IMDM (Sigma-Aldrich) supplemented with 20% FBS (Sigma-Aldrich). For expansion, the non-adherent cells were collected after 48 h, washed and replated onto a fibronectin-coated 6-well plate with the complete medium containing IMDM supplemented with 10% FBS, 10 ng/ml VEGF, 4 ng/ml fibroblast growth factor-2 (FGF-2) and 10 ng/ml EGF (US Biologicals, United States) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Medium change was done every 3 days. Adherent EPC-colonies were stained for the uptake of DiI-labeled acetylated low-density lipoprotein (acLDL, Invitrogen, United States) and binding of FITC-conjugated Ulex europaeus agglutinin I (UEA-1, Sigma)

**TABLE 1 |** List of primers.

| Gene | Forward Primer | Reverse Primer |
|---|---|---|
| VEGFA | ACCTCCACCATGCCAAGT | TAGTTCCCGAAACCCTGA |
| bFGF | CCAGTTGGTATGTGGCACTG | CAGGGAAGGGTTTGACAAGA |
| VEGFR2 | GTGATTGCCATGTTCTTCTGGC | TCAGACATGAGAGCTCGATGCT |
| CXCR4 | TCCTGCCCACCATCTATTTTATC | ATGATATGCACAGCCTTACAT |
| GAPDH | CTGCACCACCAACTGCTTAC | CAGAGGTGCCATCCAGAGTT |

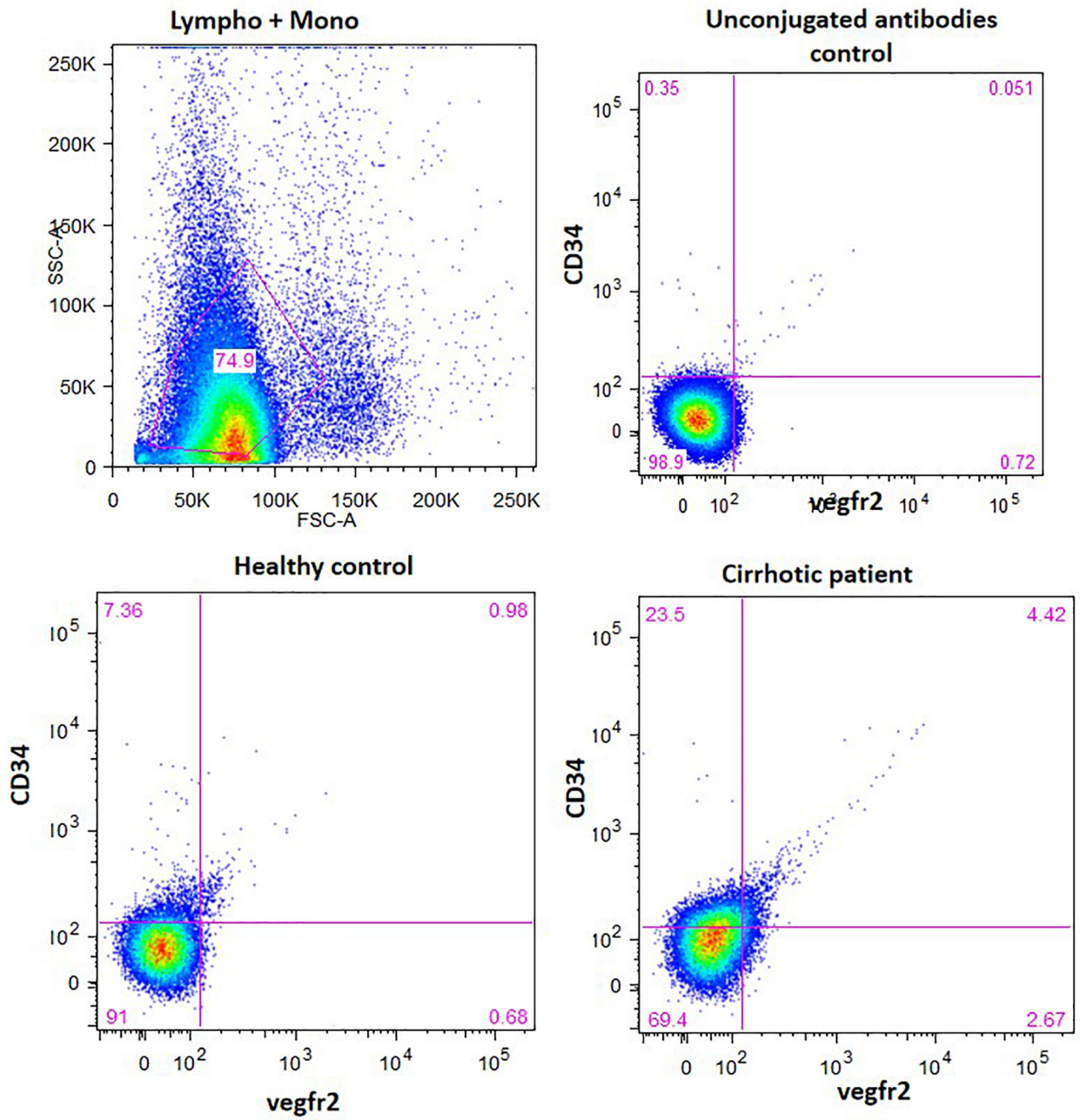


**FIGURE 2 |** EPC enumeration in whole blood by measuring the percentage of CD34 and vergfr2 dual positive cells in the lymphocyte and monocyte gated cells. The figure shows dot plots of dual stained cells in healthy controls and cirrhotic patients. Unconjugated antibodies without flourophores were used as controls.

after 7 days of cell culture as earlier described (Garg et al., 2017). The stained cells were visualized under an inverted Nikon fluorescent microscope. The identity of human EPCs at day 8 of culture was also confirmed by immunofluorescence using rabbit anti-human VEGFR2 and CD34 antibodies (1: 200, **Supplementary Table S2**, Santa Cruz Biotechnology, Santa Cruz, CA, United States).

The adherent cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min. After washing, non-specific binding was blocked with 2% BSA in PBS for 10 min. Primary antibody diluted in PBS containing 1% BSA was then added and incubated for 60 min. The cells were washed thrice with PBS and incubated for 30 min with diluted secondary antibody conjugated with a rhodamine-conjugated anti-rabbit antibody (1:500). After washing, the cells were counterstained with Ho33342 dye for 5–10 min. The cells were further washed in PBS for 5 min, mounted on a glass slide and examined under a Nikon fluorescence microscope.

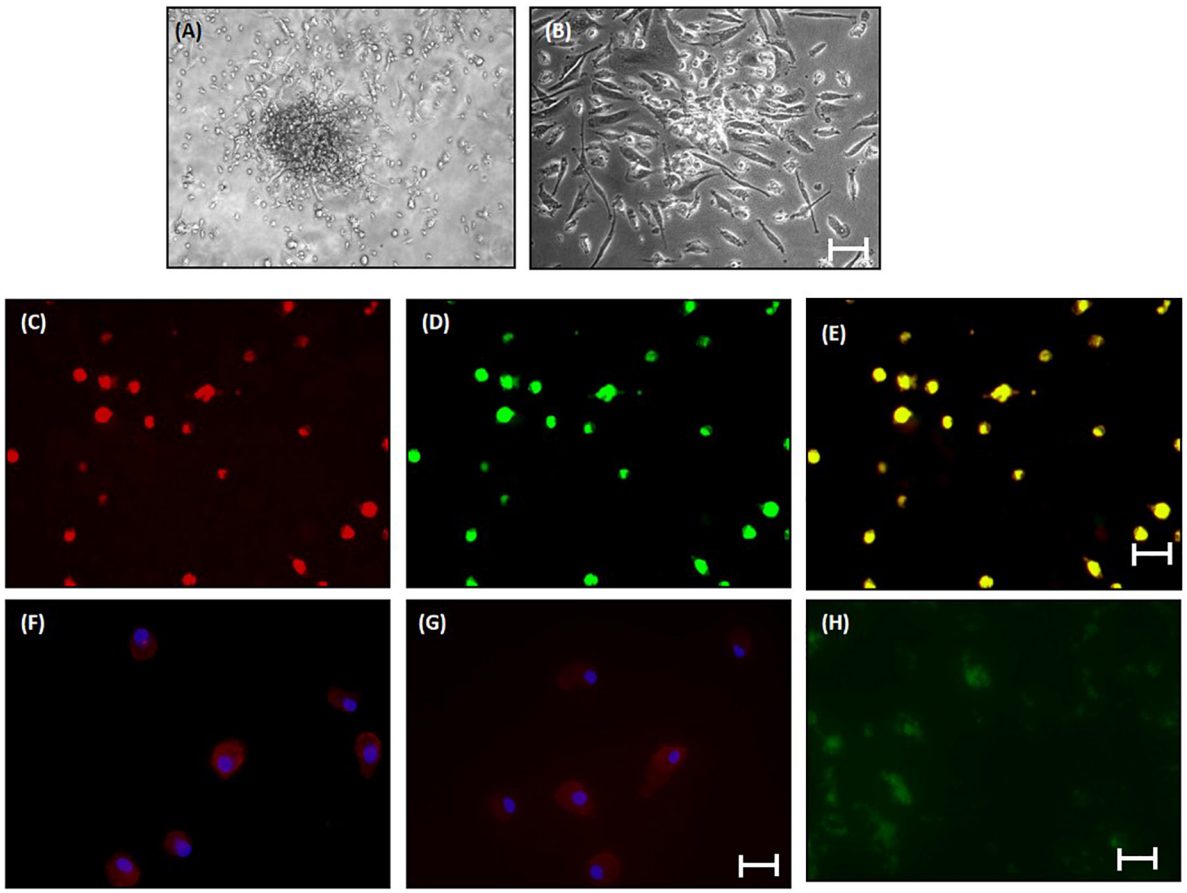


**FIGURE 3 |** Characterization of human EPCs from healthy controls (20x) **(A)** and **(B)** phase-contrast micrograph of culture-enriched endothelial progenitor cells (EPC-CFUs) on 7th day and 9th day in controls. **(C)** DiI-acLDL uptake by EPCs. **(D)** FITC-UEA lectin binding by EPCs (explained in the section "Materials and Methods"). **(E)** Overlay of **(C)** and **(D)**. **(F)** Immunofluorescence characterization of cultured EPCs with anti-human VEGFR2. **(G)** Anti-human CD34. Cells were counterstained with Hoechst dye. **(H)** *In vivo* localization of CFSE-labeled control EPCs in liver tissue sections after 24 h of transplantation.

## Cell Labeling

To detect the transplanted EPCs in cirrhotic animal livers, cells were pre-labeled with a green fluorescent marker, carboxyfluorescein succinimidyl ester (CFSE, Sigma Aldrich). Briefly, after thorough washing with DMEM (without FBS), $1 \times 10^6$ cells/500 μl cells were labeled with 10 μM CFSE and incubated at 37°C for 15 min in a water bath.

## Transplantation of EPCs

After 7 days of culture in proper growth conditions as described above, adherent EPCs from cirrhotic patients and healthy controls ($2 \times 10^6$ cells) were trypsinized and suspended in 500 μl of PBS and transplanted in BDL rats ($N = 8$ each group) after 2 weeks of ligation intravenously through tail vein thrice a week for 2 weeks. Only saline was transplanted in the control group ($N = 8$) (**Figure 1**).

In a separate set of experiments, transplanted EPCs were traced in the hepatic tissues; EPCs from healthy controls were labeled with CFSE and then transplanted into cirrhotic rats. These rats were sacrificed after 1 week of EPC transplantation, and CFSE labeling was analyzed in liver tissues for the detection of EPCs.

## Evaluation of Hepatic Fibrosis

Livers from rats of all the groups were collected after cell transplantation, fixed in 10% formalin, embedded in paraffin wax, and thin sections measuring 2.5–3 μm in thickness were prepared. Sections were stained with hematoxylin–eosin and Masson's trichrome for quantification of hepatic fibrosis. Pictures were taken and analyzed using a microscope equipped with a digital camera. Eight fields were randomly selected, and fibrosis grading was assigned by a third person blindly in all the groups.

## Gene Expression Analysis by Real-Time PCR

Total RNA from the liver tissues was isolated by using Nucleopore kit (Genetix Biotech Asia Pvt Ltd., India) as per manufacturer's instructions. RNA was quantified at 260/280 nm with Thermo Scientific Nanodrop 2000 Spectrophotometer. First strand cDNA was synthesized from 1 μg of total RNA with

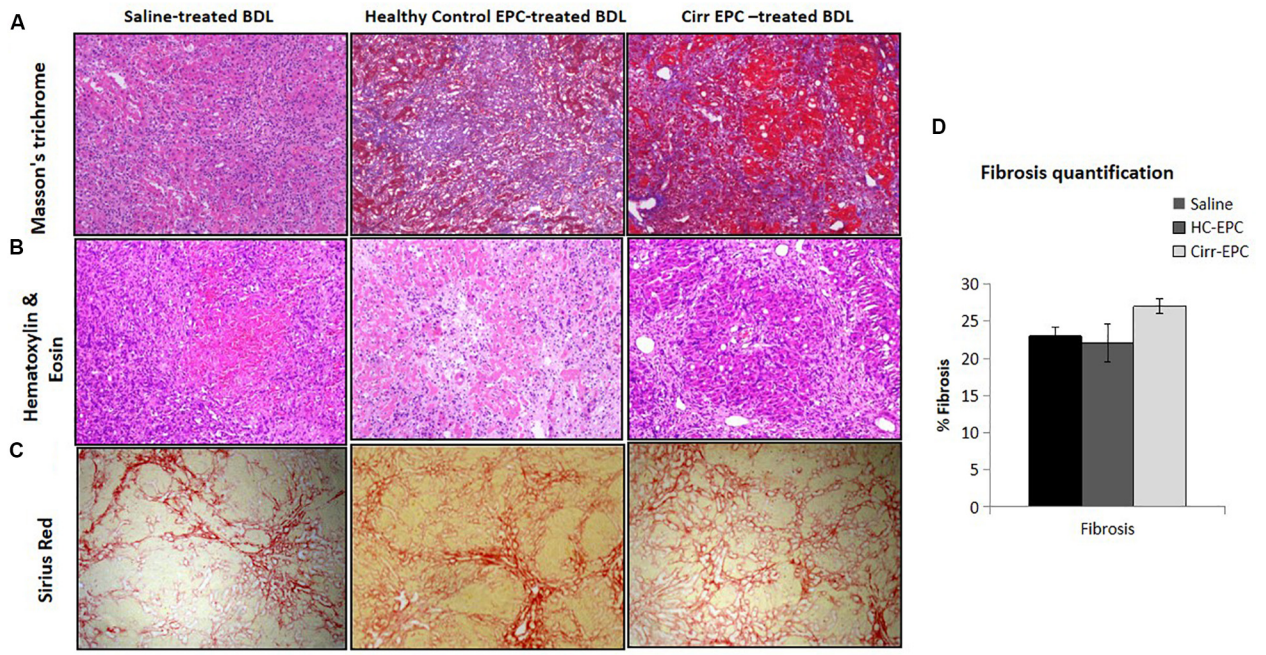


**FIGURE 4 |** Assessment of liver fibrosis (10x) in saline-treated BDL, healthy control (HC), and cirrhotic (cirr) EPC-transplanted BDL rats by **(A)** Masson's trichome staining, **(B)** H and E staining, **(C)** Sirus red staining, and **(D)** fibrosis quantification.

reverse transcriptase (Thermo Scientific Verso cDNA synthesis kit) according to the manufacturer's instructions. Quantitative real-time PCR was carried with SYBR green PCR master mix (Fermentas Life Sciences) on the ViiA7 PCR system (Applied Biosystems, United States). The following cycling parameters were used: start at 95°C for 5 min, denaturing at 95°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s, and a final 5 min extra extension at the end of the reaction to ensure that all amplicons were completely extended and repeated for 40 amplification cycles. Relative quantification of expression of relevant genes was done using the $\Delta\Delta$Ct method after normalization to the expression of the housekeeping gene, GAPDH. The genes and primer pairs are given in **Table 1**.

## *In vivo* Hemodynamic Analysis

All rats had free access to food and water until 12 h before the study. Methods for the hemodynamic evaluation in portal hypertensive rat models have been extensively described in previous studies (Kountouras et al., 1984). Briefly, animals were anesthetized and the body temperature was maintained at 37 ± 0.5°C. Portal pressure (PP; mmHg; ileocolic vein), mean arterial pressure (MAP, mmHg; femoral artery), portal blood flow (PBF; mL/min; portal vein as close as possible to the liver), and superior mesenteric artery blood flow (SMABF; mL/min; superior mesenteric artery) were estimated by perivascular ultrasonic transit-time flow probes connected to a flow meter (Transonic Systems, Ithaca, NY, United States) and recorded by a PowerLab data acquisition and analysis apparatus (8/35). Data were analyzed by the Chart v5.01 software (AD Instruments). Hepatic vascular resistance (HVR, mmHg/mL·min·g$^{-1}$) was calculated. At the end of the hemodynamic study, serum samples from all the rats were collected from inferior vena cava (IVC) for subsequent biochemical analysis.

## Serum Biochemical Analysis of Liver Function

At the time of sacrifice, serum samples from EPC transplanted BDL rats as well as from saline-treated BDL rats were collected from IVC to further evaluate alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and microalbumin levels by the standard hospital protocols.

## Immunohistochemical Staining

The liver tissue sections were fixed in 10% buffered formalin solution for 24 h, embedded in paraffin wax, and thin sections measuring 2.5–3 μm in thickness were prepared. They were deparaffinized with xylene following gradual hydration with alcohol series. They were thoroughly rinsed with running tap water. Antigen retrieval was performed with citrate buffer/Tris EDTA (pH 6 and 9). Blocking of endogenous peroxidase was done in 3% hydrogen peroxide ($H_2O_2$)/$H_2O_2$ containing buffered solution of casein and sodium azide (pH 7.6) for 10 min at room temperature to avoid non-specific binding of secondary antibodies. Intrinsic peroxidase was inactivated for 10 min with 3% $H_2O_2$ and rinsed with Tris buffered substrate (TBS, 1/15 mol/l, pH 7.6).

The sections were incubated overnight at 4°C with α-SMA (BioGenex, United States, pH 6, Ready to use), CD-31 (BioGenex, United States, pH 6, Ready to use),

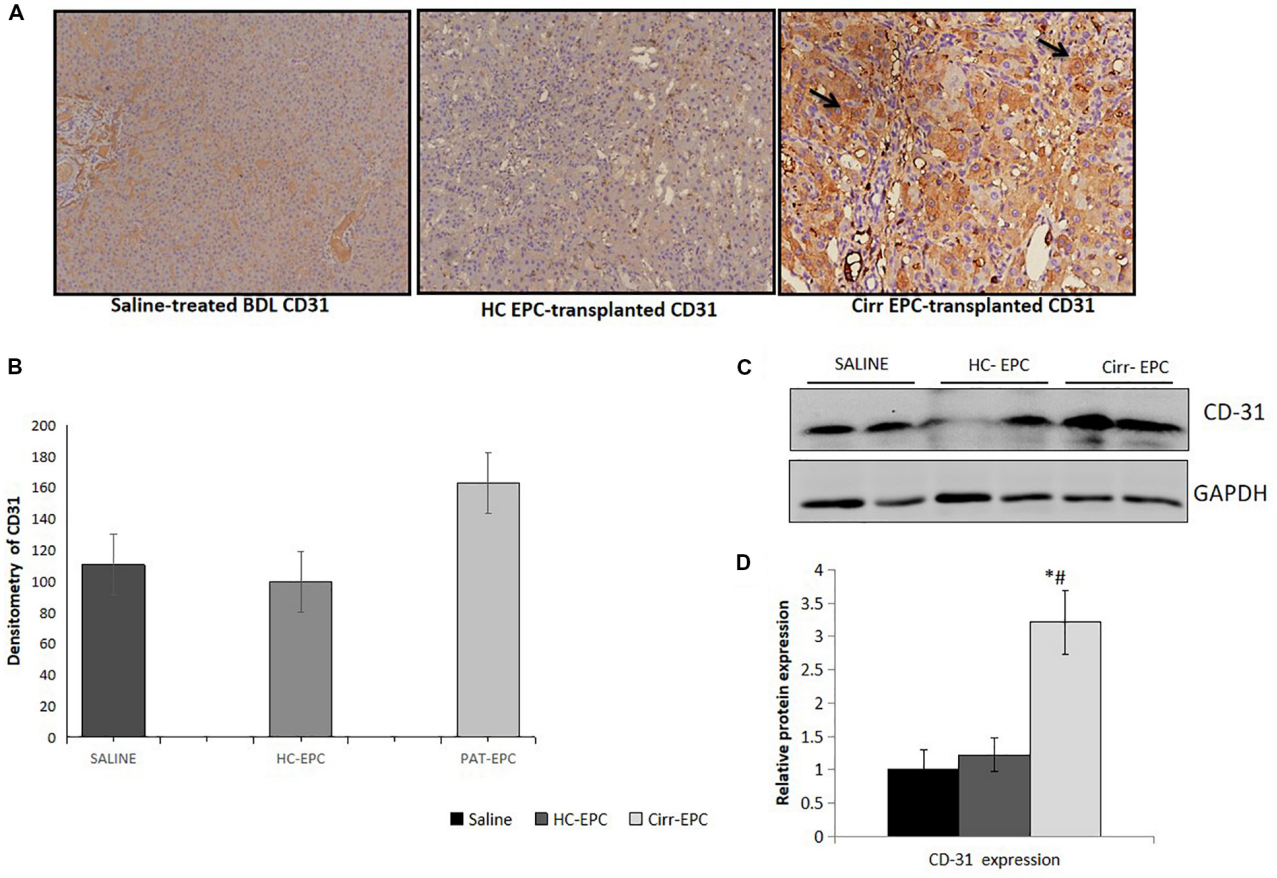


**FIGURE 5 |** Histochemical staining of liver tissues sections (10x) in saline-treated, healthy (HC), and cirrhotic (PAT) EPC-transplanted BDL rats for angiogenic markers. **(A)** CD31, **(B)** Densitometric analysis of CD31, **(C)** Representative western blots of CD31, and **(D)** Densitometric analysis of western blot in saline-treated, control, and cirrhotic EPC-transplanted BDL rats ($N = 8$). *$P < 0.05$ vs. saline, #, vs. HC-EPC.

TGF-β (Santa Cruz Biotechnology, United States, 1:200) as primary antibodies (**Supplementary Table S2**) followed by a reaction for 30 min at 20°C using a biotinylated secondary antibody (Super Sensitive polymer HRP IHC Detection System, BioGenex, Fremont, United States). After washing, the tertiary antibody (Super Sensitive polymer HRP IHC Detection System, BioGenex, Fremont, United States) was used for 20 min. Sections were rinsed first with TBS and then under running tap water. Then, mixed solution of 3, 3-diaminobenzidine tetra hydrochloride (DAB) substrate (in dark) was used for color development (visualization) of the reaction product. Sections were further counterstained with hematoxylin for 1 min, dehydrated and mounted with DPX and observed under the microscope.

## Protein Expression Analysis by Western Blotting

Samples of shock-frozen livers were homogenized in a buffer containing 25 mM Tris/HCl, 5 mM ethylenediamine tetraacetic acid, 10 μM phenylmethanesulfonyl fluoride, 1 mM benzamidine, and 10 μg/mL leupeptin. Samples were diluted with sample buffer. Determination of proteins in the homogenates was performed with Bradford (Sigma) and pre-diluted protein assay standards (BSA set kit, thermo scientific, United States). Samples (50 μg of protein/lane) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 15% gels) for α-SMA and CD31, and proteins were blotted on PVDF membranes charged with methanol. To ensure equal protein loading, Ponceau-S staining was performed. The membranes were blocked with 5% BSA, incubated with primary antibodies α-SMA (1:1000) and CD31 (1:200) and thereafter with corresponding secondary peroxidase-coupled antibodies (**Supplementary Table S2**). Blots were developed with enhanced chemiluminescence Pierce, ECL plus western blotting substrate (Thermo scientific, United States). Intensities of the resulting bands on each blot were compared densitometrically with image J software.

## Statistical Analysis

Using SPSS software, statistical analysis was performed. Results are expressed as mean ± standard deviation. Comparisons between groups were performed with the Student's *t*-test for

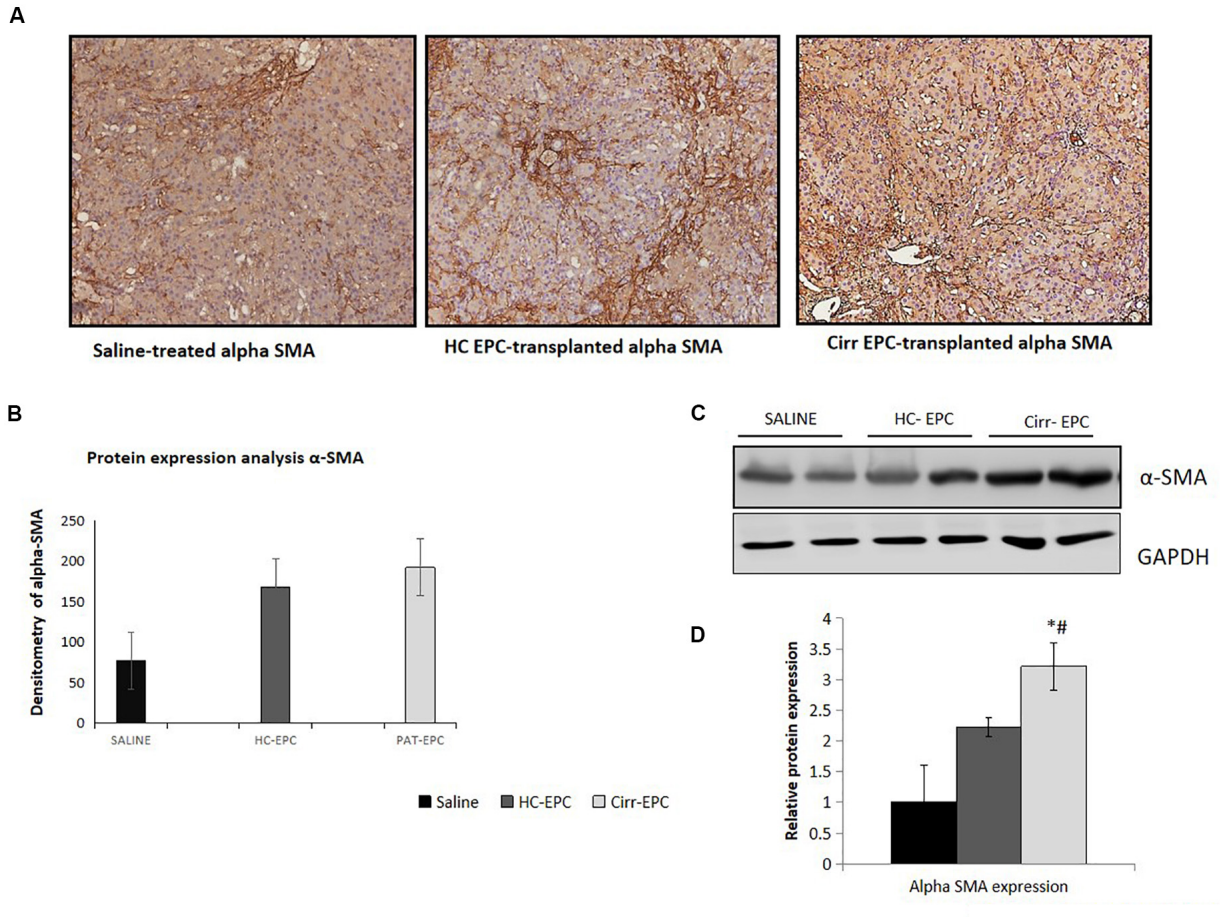


**FIGURE 6 |** Histochemical staining of liver tissues sections (10x) in saline-treated, healthy control, and cirrhotic EPC-transplanted rats for fibrosis markers. **(A)** Alpha-SMA, **(B)** Densitometric analysis of alpha-SMA, **(C)** Representative western blots of alpha-SMA, and **(D)** Densitometric analysis of western blots of alpha-SMA in saline-treated, control, and cirrhotic EPC-transplanted rats. *$P < 0.05$ vs. saline, #, vs. HC-EPC.

unpaired data. Differences were considered significant at a $P$-value $< 0.05$.

# RESULTS

## Characterization of Human EPCs *in vitro* and *in vivo*

The percentage of CD34-vegfr2 dual positive EPCs in blood was increased in patients with cirrhosis as compared to the healthy subjects (**Figure 2**). At day 2–3, large round adherent cells started appearing from plated human PBMCs, which then became spindle-shaped at around day 7–9 (**Figures 3A,B**). The attached cells after day 7 stained positive for DiI-acetylated LDL (red fluorescence) and FITC-UEA-1 lectin (green fluorescence) (**Figures 3C–E**). After 7 days in culture, the adherent cells also stained positive for specific EPC surface proteins, vegfr2 (**Figure 3F**) and CD34 (**Figure 3G**). EPCs from patients with cirrhosis also behaved in a similar fashion in culture (results not shown).

*In vivo*, EPCs labeled with CFSE (green fluorescence) were observed surrounding the portal tracts, fibrous septa, and hepatic lobules in the BDL rats, 24 h after EPC transplantation (**Figure 3H**). We did not observe any infiltrated cells with green fluorescence in the saline-infused BDL rat livers (data not shown).

## Collagen Deposition in EPC-Transplanted BDL Rats

In saline-treated BDL rats, hepatic necrosis and mild fibrosis with some viable inflammatory cells were observed. Peribiliary and interstitial collagen deposition was evident in all groups of BDL rats, as shown by positive Masson's trichrome staining (**Figures 4A,B**). There was an increase of portal fibrosis (grade 3) to marked cirrhosis (grade 4) in cirrhotic-EPC-treated rats as compared to healthy EPC-treated and saline-treated BDL rats (**Figures 4A–D**). Liver collagen staining as determined by Sirius red was less in the liver in saline-treated and healthy control-EPC treated rats in comparison with cirrhotic-EPC-treated rats (**Figures 4C,D**).

## Intrahepatic Angiogenesis and Fibrosis in EPC-Transplanted BDL Rats

The expression of CD31 was less in healthy EPC- and saline-treated rat liver sections in both immunohistochemistry and western blot studies. There was an increase in the expression of LSEC differentiation and angiogenesis marker, CD31 in cirrhotic EPC-treated rats as compared to healthy EPC- and saline-treated rats ($P < 0.05$ for each). In cirrhotic EPC-transplanted rats, the expression of CD31 was majorly seen in portal areas/peri-portal regions and fibro-septae suggesting neovascularization in these areas (**Figures 5A–D**).

Increased α-SMA positive cells paralleled the development of increased fibrosis in BDL rats. Histochemical and densitometric analysis of the western blots showed that the expression of the fibrosis marker, alpha-SMA present on activated HSCs was significantly higher in cirrhotic EPC-treated rats as compared to the saline- and healthy-EPC treated rats ($P < 0.05$ for each) (**Figures 6A–D**). The expression of TGF-β, a fibrosis marker, was also increased in the liver sections of cirrhotic EPC-treated rats and control-EPC treated rats as compared to the saline-treated rats (**Supplementary Figure S1**). To ascertain if patient EPCs specifically activated HSCs in culture, we set up co-cultures between HSC cell lines (LX2) and conditioned media (CM) from EPCs (both healthy and cirrhotic). Results showed that in the presence of patient EPCs, there was a maximum proliferation of LX2 cells, much higher than that observed with control EPCs or negative control (media only) (**Supplementary Figures S3A,B**). Also secretion of an important angiogenic factor, basic fibroblast growth factor (bFGF), was significantly higher in HSCs co-cultured in the presence of CM from cirrhotic patients' EPCs in comparison to that with HSCs with CM from control or healthy EPCs (**Supplementary Figure S3C**).

## Hepatic Hemodynamics in EPC-Transplanted BDL Rats

Bile duct ligation cirrhotic animals exhibited portal hypertension when compared to control rats (**Table 2**). The BDL cirrhotic rats transplanted with cirrhotic EPCs exhibited statistically significant higher portal pressure than rats transplanted healthy EPCs or receiving vehicle (17.2 ± 2.1 vs. 13.8 ± 2.2 mmHg; +25%; $P = 0.003$). We did not observe any change in PP in healthy EPC transplanted rats in comparison with vehicle treated rats (14.5 ± 2.1 vs. 13.8 ± 2.2 mmHg; +5%; $P = 0.91$). Also, we did not observe any change in PBF. The increment in PP was not associated with change in PBF, thus suggesting that portal hypertension aggravation in cirrhotic EPC transplanted rats derived from an increment in the HVR (cirrhotic EPCs rats: +25%) in comparison with healthy EPC transplanted rats ($P < 0.05$). MAP, SMABF, and HR were not modified by cell transplantation (**Table 3**).

## Liver Functions in EPC-Treated BDL Rats

Serum from the untreated and EPC-treated rats was collected for the analysis of liver functions including the estimation of the levels of urea, total bilirubin, glucose, micro albumin, and alanine transaminase. We did not observe any significant difference in the levels of urea, bilirubin, glucose, albumin, ALT, and AST in EPC-treated BDL rats as compared to saline-treated BDL rats (**Supplementary Figure S2**).

## Angiogenic Gene Expression in EPC-Treated BDL Rats

Next, we studied the effect of healthy and cirrhotic EPC transplantation on angiogenic gene expression in the liver tissues. With respect to saline-treated rats, the expression of VEGFA gene was markedly upregulated in the livers of both healthy and cirrhotic EPC transplanted rats. However, the expression of other genes including VEGFR2, bFGF, and CXCR4 was enhanced

**TABLE 2 |** Hemodynamic parameters.

| Characteristics hemodynamic parameters | Experimental groups | | |
|---|---|---|---|
| | Healthy control | BDL Veh | *P*-value |
| *N* | 6 | 8 | – |
| MAP (mmHg) | 113 ± 10 | 74 ± 9 | $P < 0.05$ |
| PP (mmHg) | 7.5 ± 0.9 | 14.5 ± 2.1 | $P < 0.05$ |
| PBF (ml/min) | 14.2 ± 3.6 | 18.9 ± 8.6 | $P < 0.05$ |
| HVR (mmHg/ml·min·g$^{-1}$) | 2.5 ± 0.6 | 22.5 ± 14.3 | $P < 0.05$ |
| HR (beats/min) | 354 ± 49 | 345 ± 33 | NS |
| Liver weight | 11 ± 3.2 | 25 ± 6.6 | $P < 0.05$ |
| Body weight | 340 ± 65 | 410 ± 95 | NS |

**TABLE 3 |** Hemodynamic parameters.

| Characteristics hemodynamic parameters | Experimental groups | | | |
|---|---|---|---|---|
| | BDL Veh | BDL + Healthy EPC | BDL + Cirrhotic EPC | *P*-value |
| *N* | 8 | 8 | 8 | – |
| MAP (mmHg) | 74 ± 9 | 68 ± 9.7 | 71 ± 28 | NS |
| PP (mmHg) | 14.5 ± 2.1 | 13.8 ± 2.2 | 17.2 ± 2.1 | $^{*\#}P < 0.05$ |
| PBF (ml/min) | 18.9 ± 8.6 | 17.1 ± 4.8 | 20.6 ± 7.2 | NS |
| HVR (mmHg/ml·min·g$^{-1}$) | 22.5 ± 14.3 | 20.1 ± 7.2 | 25.6 ± 13 | $^{*\#}P < 0.05$ |
| HR (beats/min) | 345 ± 33 | 333 ± 147 | 426 ± 37 | NS |
| Liver weight | 25 ± 6.6 | 27.6 ± 8.4 | 27.4 ± 4.9 | NS |
| Body weight | 410 ± 95 | 398 ± 134 | 401 ± 75 | NS |

*$^{*}P < 0.05$ Cirrhotic EPC vs. Veh; $^{\#}P < 0.05$ Cirrhotic EPC vs. Healthy EPC.*

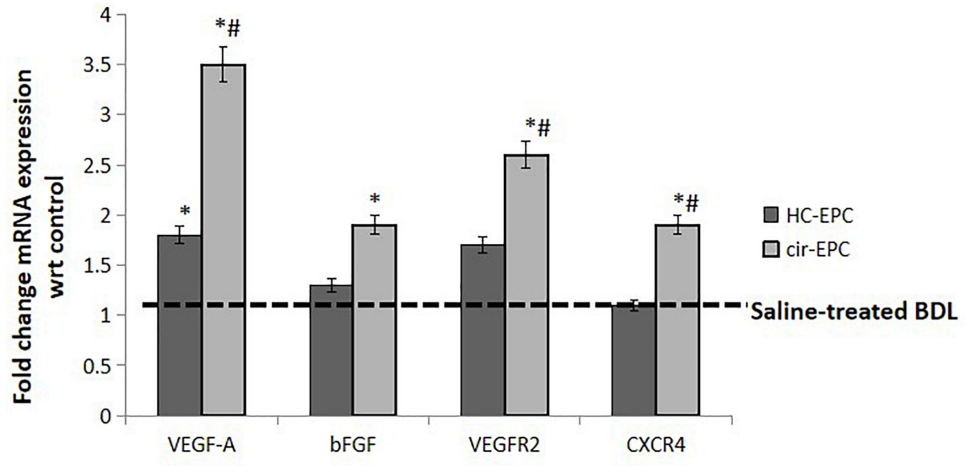


**FIGURE 7 |** mRNA expression of genes in liver tissue samples of healthy and cirrhotic EPC-transplanted rats. Dotted line represents control showing gene expression in Huh7 cells treated with BSA. Data represent mean ± SD, ($n$ = 4). *$P$ < 0.05 vs. saline, #$P$ < 0.05 vs. healthy EPC-treated rats.

only in cirrhotic EPC-transplanted rats in comparison with both saline- and healthy EPC-treated rats (**Figure 7**).

## DISCUSSION

Endothelial progenitor cells have been implicated in both injury and repair. Previous studies have highlighted the regenerative role of healthy EPCs during liver injury. These studies have shown their role in restoring vascular density and promoting hepatic regeneration in the injured livers (Fadini et al., 2012; Nakamura et al., 2012). However, as angiogenesis plays a pathogenic role in fibrosis, we hypothesized that EPCs would be pro-fibrotic as well (Medina et al., 2004, 2005; Ding et al., 2014; Tripathi et al., 2018). Our previous studies have demonstrated that during liver injury, endogenous bone marrow EPCs migrate to the liver and show strong correlation with liver fibrosis (Kaur et al., 2012). In this study, we evaluated the *in vivo* effects of healthy and cirrhotic EPCs in liver injury. We demonstrate that transplantation of cirrhotic EPCs (CD34+ vegfr2+) in BDL rats aggravates hepatic angiogenesis, fibrosis, and portal hypertension possibly via their interaction with LSECs and HSCs (**Figure 8**).

Our results showed an increase in CD31-postive blood vessels and angiogenesis in BDL models in comparison with the control rats as has been reported earlier (Fadini et al., 2012; Nakamura et al., 2012). There was a further increase in the number of CD31-positive blood vessels in cirrhotic EPC-treated BDL rats as compared to healthy EPC- and saline-treated BDL rats. Healthy liver vessels or LSECs are fenestrated and normally do not express CD31. An increased expression of CD31 is indicative of LSEC differentiation and plays a pivotal role in the activation of HSCs (Lee and Friedman, 2011). We also observed a significant enhancement in the hepatic expression of angiogenic growth factors and receptors such as VEGF, bFGF, and receptors including VEGFR2 and CXCR4 in cirrhotic EPC-treated BDL rats as compared to healthy EPC-treated rats. This suggests that the proangiogenic activity of cirrhotic EPCs is excessive and may induce the formation of abnormal vessels in the already cirrhotic liver via upregulation of signals such as VEGF and bFGF. Our results corroborate the findings of earlier studies that bFGF, VEGF, and VEGFR2 play key roles in the angiogenesis of the cirrhotic liver (Rosmorduc et al., 1999; Medina et al., 2005; Lee and Friedman, 2011; Xie et al., 2012). Our previous study also demonstrated that cirrhotic EPCs secrete higher levels of bFGF and VEGF in culture and exhibit greater angiogenic impact on LSECs in culture. It has also been shown earlier that a predominance of CXCR4 expression of LSECs shifts the pro-regenerative response toward the pro-fibrogenic response during liver injury (Medina et al., 2004). Activated LSECs are known to modulate HSCs that then mutually promote the function of each other, leading to liver fibrosis (Lee and Friedman, 2011). We observed a significant increase in the fibrogenic markers, α-SMA and TGF-β in cirrhotic-EPC treated BDL rats, as compared to the saline- and healthy EPC-treated rats, suggesting that the formation of abnormal vessels by cirrhotic EPCs facilitates liver fibrogenesis or even vice versa. CM from cirrhotic EPCs also significantly enhanced the proliferation of LX2 cells and secretion of angiogenic factor in culture more than the healthy EPCs, suggesting their direct role in HSC activation. This observation validates our previous study, where we reported that CM from EPCs increased the expression of alpha-SMA in the cultured mouse HSCs (Kaur et al., 2012).

An increase in liver angiogenesis and fibrosis causes deregulation of the hepatic hemodynamics and correlates positively with portal hypertension (Poisson et al., 2017; McConnell and Iwakiri, 2018). Our results demonstrated that pro-angiogenic cirrhotic EPCs markedly affect the vascular physiology of the liver (Gracia-Sancho et al., 2019). They enhanced the portal pressure favoring hypercontractility state, leading to increased intrahepatic vascular resistance and decreased liver perfusion. A mild increase (non-significant data) in portal pressure and a decrease in hepatic vascular resistance

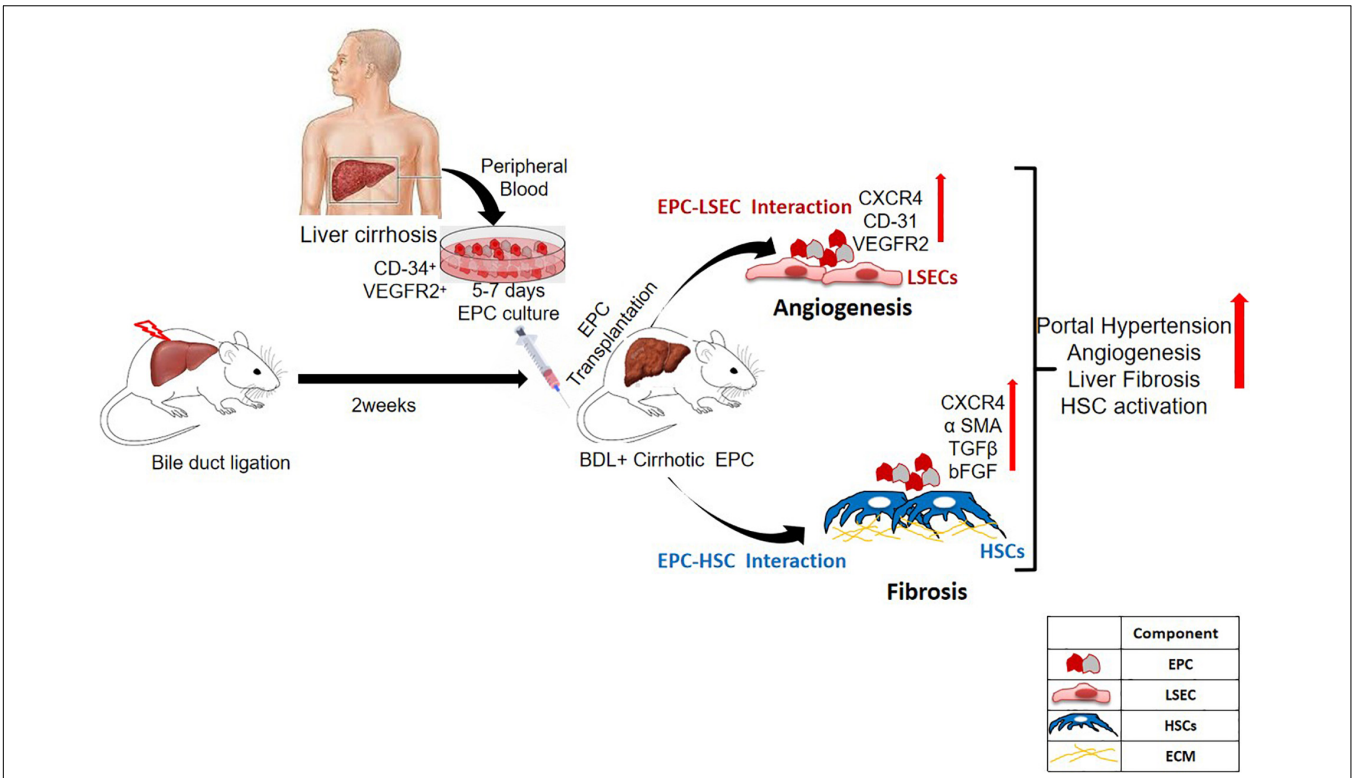


**FIGURE 8 |** Overall summary of the study. Transplantation of cirrhotic EPCs (CD34+-vegfr2+) in BDL rats interacts with LSECs and HSCs *in vivo* and increases hepatic angiogenesis, and fibrosis further aggravates portal hypertension.

were observed in healthy EPC transplanted cirrhotic models as has been reported earlier (Nakamura et al., 2007), again suggesting that healthy and cirrhotic EPCs have significant differences in their properties and that cirrhotic EPCs have a detrimental impact on portal pressures and blood flow in the liver in comparison with the healthy EPCs.

We observed no changes in the liver functions including transaminases, total bilirubin, total protein, and albumin either in healthy or cirrhotic EPC-transplanted rats. It has been, however, reported in an earlier study that normal liver function parameters are restored in EPC-transplanted $CCl_4$-treated rats (Garg et al., 2017). This may be due to the fact that we used BDL models in our study which is a combined model of hepatocyte and cholestatic liver injury. Unlike the CCl4-induced liver cirrhotic models, in the BDL model, extrahepatic cholestasis due to prolonged obstruction of bile flow results in even more extensive morphological and biochemical changes (Fadini et al., 2012). The low efficacy of healthy EPC treatment in our study may be attributed to an increased degree of fibrosis and deposition of extracellular matrix proteins in the BDL rats in comparison with the CCl4-treated rats. A limitation of our study is that we have transplanted human EPCs into rats. Although the adaptive immune functions of BDL rats are highly immunocompromised, these rats elicit strong innate immune responses such as activation of neutrophils, macrophages, and natural killer cells which are currently being recognized as important components in xenograft rejection (Chaignaud et al., 1994). Another limitation of the study is the small sample number of animals used to perform adequate statistical comparisons. Also, we have used a very heterogeneous group of CD34-vegfr2-positive EPCs for transplantation, and hence, it is difficult to interpret the true relevance of angiogenic EPCs (which may be a small subset) to liver fibrosis and/or improvement in functions (Fadini et al., 2012).

Overall, this study concludes that cirrhotic EPCs have enhanced angiogenic and profibrogenic functions *in vivo* as compared to the healthy control EPCs. Our findings imply that the endogenous mobilization of cirrhotic EPCs in cirrhotic patients may lead to fibrosis due to enhanced proangiogenic activity of EPCs. Hence, these patients are likely to benefit from therapies which are aimed at lowering the numbers of proangiogenic EPCs.

## DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material**.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Ethics Committee, ILBS.

## AUTHOR CONTRIBUTIONS

DT, MH, and HS performed all animal experiments, western blotting, and analyzed the data. DT did all the hemodynamic experiments and analysis. DT and MH wrote the manuscript. IK and CB performed immunohistochemistry of the liver tissues. PR performed RT-PCRs and data analysis. SK designed the study, performed the data analysis along with DT, and finalized the manuscript. SS gave inputs in designing of the study and editing of the manuscript.

## FUNDING

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00617/full#supplementary-material

**FIGURE S1 |** Immunohistochemical staining of liver tissues sections in saline-treated, control, and cirrhotic EPC-transplanted rats for TGF-β.

**FIGURE S2 |** Histograms showing the effect of saline, control, and cirrhotic EPCs treatment on liver functions in BDL rats.

**FIGURE S3 | (A)** Representative images (10×) of LX2 cells (HSCs) incubated with conditioned media (CM) from healthy (control) and cirrhotic patient EPCs. **(B)** Bar diagram showing absorbance of MTT assay depicting cell proliferation in different conditions. **(C)** Absorbance showing FGF levels in cells alone and co-cultured cells.

**TABLE S1 |** Characteristics of patients used in the study.

**TABLE S2 |** List of Antibodies used in the study.

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REVIEW
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# Prominent Receptors of Liver Sinusoidal Endothelial Cells in Liver Homeostasis and Disease

*Ekta Pandey, Aiah S. Nour and Edward N. Harris**

*Department of Biochemistry, University of Nebraska, Lincoln, NE, United States*

Liver sinusoidal endothelial cells (LSECs) are the most abundant non-parenchymal cells lining the sinusoidal capillaries of the hepatic system. LSECs are characterized with numerous fenestrae and lack basement membrane as well as a diaphragm. These unique morphological characteristics of LSECs makes them the most permeable endothelial cells of the mammalian vasculature and aid in regulating flow of macromolecules and small lipid-based structures between sinusoidal blood and parenchymal cells. LSECs have a very high endocytic capacity aided by scavenger receptors (SR), such as SR-A, SR-B (SR-B1 and CD-36), SR-E (Lox-1 and mannose receptors), and SR-H (Stabilins). Other high-affinity receptors for mediating endocytosis include the FcγRIIb, which assist in the antibody-mediated removal of immune complexes. Complemented with intense lysosomal activity, LSECs play a vital role in the uptake and degradation of many blood borne waste macromolecules and small (<280 nm) colloids. Currently, seven Toll-like receptors have been investigated in LSECs, which are involved in the recognition and clearance of pathogen-associated molecular pattern (PAMPs) as well as damage associated molecular pattern (DAMP). Along with other SRs, LSECs play an essential role in maintaining lipid homeostasis with the low-density lipoprotein receptor-related protein-1 (LRP-1), in juxtaposition with hepatocytes. LSECs co-express two surface lectins called L-Specific Intercellular adhesion molecule-3 Grabbing Non-integrin Receptor (L-SIGN) and liver sinusoidal endothelial cell lectin (LSECtin). LSECs also express several adhesion molecules which are involved in the recruitment of leukocytes at the site of inflammation. Here, we review these cell surface receptors as well as other components expressed by LSECs and their functions in the maintenance of liver homeostasis. We further discuss receptor expression and activity and dysregulation associated with the initiation and progression of many liver diseases, such as hepatocellular carcinoma, liver fibrosis, and cirrhosis, alcoholic and non-alcoholic fatty liver diseases and pseudocapillarization with aging.

**Keywords: liver, sinusoidal endothelial cells, scavenger receptors, cell surface receptor, endocytosis, ligand binding**

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****Correspondence:***
*Edward N. Harris*
*eharris5@unl.edu*

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## INTRODUCTION

The liver is considered a crucial organ of the body due to its involvement in numerous processes, such as metabolism, immunity, detoxification, nutrient storage, among others. The liver is composed primarily of four distinct cell types, differentiated into two categories as parenchymal cells (PC, 60–80%) and non-parenchymal cells (NPC 20–40%). The NPC population is composed of liver sinusoidal endothelial cells/LSECs (50%), Kupffer cells/KCs (20%) and stellate cells (<1%). The remaining NPCs are composed of lymphocytes (25%) and biliary cells (5%) (Racanelli and Rehermann, 2006). The role of hepatocytes, KCs and stellate cells in maintaining liver homeostasis is well documented. However, the LSEC, is the most understudied due to technical challenges in purification and culturing *ex vivo*.

Prof. Eddie Wisse first proposed the ultrastructure of liver sinusoidal endothelial cells (LSEC) in 1970, which differentiated LSECs from KCs and paved the way for future study on LSECs and elucidation of their function (Wisse, 1970, 1972). A few years later, several research groups developed LSEC isolation techniques and identified their role in the uptake of various substances *in vitro* (Seglen, 1976; Smedsrod et al., 1984). In the early 1980s, LSECs were identified as a significant clearance site for blood-borne hyaluronan, which established their role as scavenger cells (Fraser et al., 1981). This led to an increase in interest among scientists from other discipline, such as immunology, virology, cancer, and more to further comprehend the various roles performed by LSECs. This review focuses on the detailed description of LSEC morphology and their scavenger, adhesion and other prominent receptors that define their functional roles in the hematological and hepatic systems during health and disease.

## LSEC MORPHOLOGY

Liver sinusoidal endothelial cells (LSECs) form the inner lining of liver sinusoidal blood vessels or capillary bed which serves as the site for mixing nutrient-rich blood from the hepatic portal vein and oxygen-rich blood from the hepatic artery (Sorensen et al., 2015). Here, the LSECs assist in clearing macromolecular waste (extracellular matrix material and foreign molecules) from the blood and regulate hepatic vascularity. Individual LSEC's are flat and very small in size, no thicker than 5 μm at the center and 0.3 μm at the periphery (Wisse, 1970, 1972; Smedsrod et al., 1988b; Falkowska-Hansen et al., 2007). Cytoplasmic projections, such as filopodia, lamellipodia, and microvilli are absent in LSECs, giving them a smooth appearance. LSECs contain numerous fenestrae (small open pores) that facilitate the selective exchange of molecules between the blood and underlying stellate and hepatocytes (Wisse et al., 1985; Braet and Wisse, 2002). Fenestrae are located in the cytoplasm of LSECs and usually are 50–200 nm in diameter and organized in groups known as sieve plates (Wisse et al., 1985). They are also distributed individually on the surface of endothelium or organized in a labyrinth or mesh-like structure (Taira, 1994; Braet et al., 2007, 2009; **Figure 1**). LSECs do not have a basement membrane or basal lamina and diaphragm, permitting direct access of solutes to the perisinusoidal space or the Space of Disse. The lack of a basal lamina has also been identified in several animals, such as chickens and bony fish (Fraser et al., 1986; Eng and Youson, 1992). The Space of Disse hosts the stellate cells as well as hepatocyte microvilli. During fibrosis or chronic inflammation, activated stellate cells contribute to the deposition of extracellular matrix in the Space of Disse, forming a continuous basal membrane. This new basal lamina is mostly composed of collagen that inhibits the permeability of the Space of Disse and reduces the solute exchange between the parenchyma and blood (Han et al., 2001; Mak et al., 2012).

LSEC's are unique because of their plentiful fenestrae. The LSEC fenestrae can change their diameter in response to the cellular environment (Wisse et al., 1996; Braet and Wisse, 2002). The mechanism for fenestral contraction and dilation was first discovered by researchers in the 1980s and is now known as a dynamic process (Monkemoller et al., 2015; Zapotoczny et al., 2019). Fenestrae is surrounded by actin filaments, suggesting the role of cytoskeleton is central in the formation and maintenance of fenestrae. This idea was further supported by several researchers in the successive years as immunofluorescence microscopic study substantiated the presence of actin, myosin, microtubule and calmodulin as necessary in forming these structures (Wisse, 1970; Braet and Wisse, 2002). Actin, myosin, and calmodulin influence the diameter of each pore and determine how long it persists. Serotonin has also been found to regulate contraction of fenestrae by increasing the intracellular calcium concentration (Gatmaitan et al., 1996; Yokomori, 2008; Furrer et al., 2011). Increase in the thickness of LSECs, unexpected formation of basal lamina and reduction in the number of pores is known as defenestration. Defenestration of LSECs gives rise to pseudocapillarization, a disorder evident in liver fibrosis, atherosclerosis and other aging associated diseases (Le Couteur et al., 2001, 2007). Fenestrae is maintained cooperatively by hepatocytes and stellate cells mediated paracrine and autocrine signaling (DeLeve et al., 2004). Vascular endothelial growth factor (VGEF), a hormone that functions in blood vessel growth, promotes paracrine signaling from hepatocytes and stellate cells stimulates autocrine signaling of nitric oxide (NO) from LSECs. Animals lacking VEGF tend to exhibit increased defenestration in their LSECs, suggesting that this molecule plays a mechanistic role in either their formation or maintenance (Kus et al., 2019).

Defenestrated LSECs results in the reduction of hepatic uptake of lipoproteins and is one of the causes of hyperlipoproteinemia (Fraser et al., 2012). Similarly, defenestration has also been observed to play an important role in the progression of non-alcoholic fatty liver disease (NAFLD). Miyao et al. demonstrated that since LSECs are a "gatekeeper" for the liver parenchyma, their injury during early stages of NAFLD determines the severity of subsequent injury and progression of NAFLD (Miyao et al., 2015; Shetty et al., 2018). In the past few years, some of this data has been called into question in that some have observed no difference in NAFLD and fenestration levels of LSECs and that LSECs have a more calming effect on injured hepatocytes and

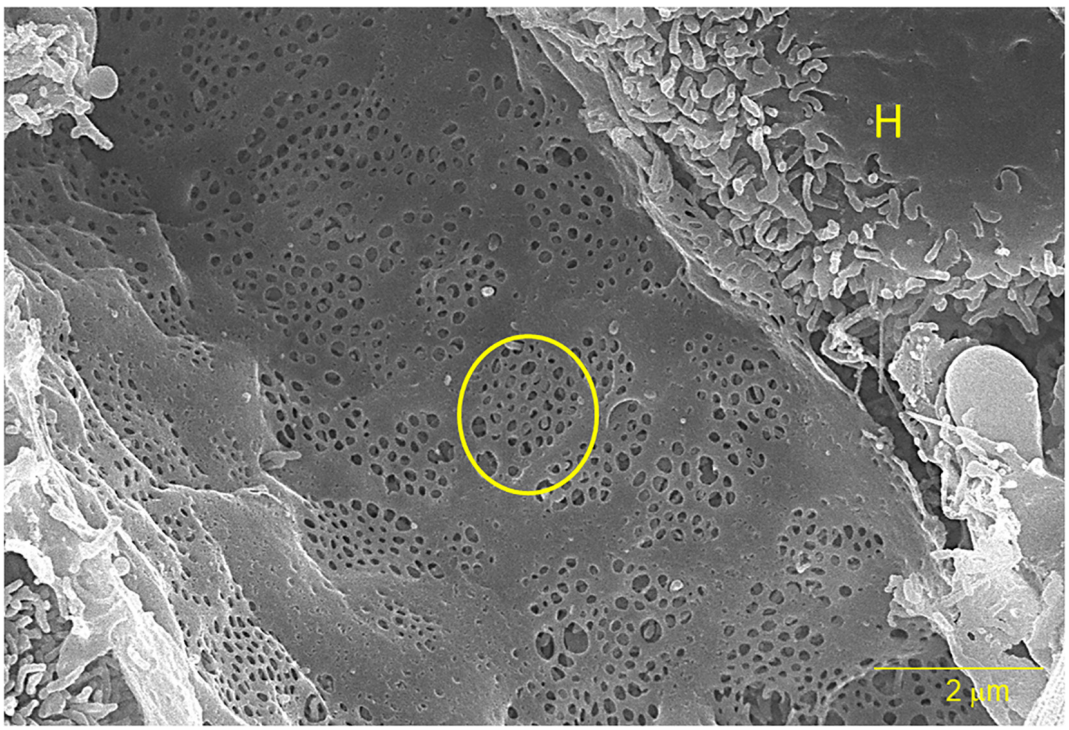


**FIGURE 1 |** A scanning electron micrograph of a sliced rat liver at 10,000× magnification. The yellow circle outlines one of several sieve plates and the "H" indicates an adjacent hepatocyte. The bar represents 2 μm.

activated stellate cells (Kus et al., 2019). This idea is reinforced in a study of autoimmune hepatitis in which human LSECs in biopsies from young people less than 20 years old were assessed for damage and the formation of a basement membrane. Defenestration did occur in about half of the subjects, but little to no basement membrane formed in the Space of Disse suggesting that LSECs have regenerative properties and may soften the assault on the liver (Lotowska et al., 2018). While there was no causative relationship proven, the jury is still out on whether progressive NAFLD is associated with defenestration.

Another salient characteristic of LSECs that differentiate them from other endothelial cells is their higher endocytic ability. LSECs only make up about 3% of total liver volume, however, they contribute to about 45% of pinocytic vesicles in the liver (Blouin et al., 1977). A study was conducted to elucidate the mechanism of cross presentation by LSECs as a link was found between endocytosis and antigen presentation (Burgdorf et al., 2008). This study reported that LSECs were most efficient at internalizing circulating antigen in the blood as compared to dendritic cells (DC) or macrophages of the spleen as well as by KC and DC of the liver (Schurich et al., 2009). LSECs are well equipped with high affinity endocytic scavenger receptors and lysosomal activity that helps in the internalization and catabolization of a large number of waste substances (**Table 1**) as well as small colloidal particles (Knook and Sleyster, 1980; Juvet et al., 1997; Kawai et al., 1998; Elvevold et al., 2008a).

Kjeken et al. (2001) showed that a clathrin-dependent mechanism is used by LSECs for fluid phase endocytosis. Furthermore, they found that LSECs have higher expression of clathrin protein and twice the number of clathrin-coated pits as compared to KC or hepatocytes. Several years later, Falkowska-Hansen et al. (2007) reported an interesting finding about clathrin-coated vesicles in primary rat LSECs. They found that clathrin heavy chain (CHC) is distributed as net-like structure which was unique to primary rat LSECs. They also showed the co-localization of CHC with microtubules. Furthermore, they found that clathrin-coated vesicle (CCV) function is dependent on microtubules as disruption of microtubules resulted in dysregulation of intracellular transport as well as aberrant signaling of organelles involved in clathrin-mediated endocytosis in LSECs (Falkowska-Hansen et al., 2007). Experiments evaluating the endocytosis capacity of primary LSECs *in vitro* must be performed the same day or within 24 h of purification of the LSECs. After this time period, endocytosis sharply decreases and ceases altogether around day 4 (Braet et al., 1994). Interestingly, the disappearance of fenestrae takes place over the same time course (Braet et al., 2005). To date, the record holder for maintaining LSECs in culture with their native endocytic capacity is the Smedsrod group using an animal-free medium which allowed endocytosis to occur out to 30 days using a pig model (Elvevold et al., 2005). It should be kept in mind that the study of endocytosis in LSECs must be performed with the freshly purified primary cells as no cell line to date expresses all of the specialized receptors nor morphological features that make these cells so dynamic.

**TABLE 1 |** Receptors expressed by LSECs and their known ligands.

| Receptor | Ligands | References |
|---|---|---|
| SR-A1/SR-A1.1 | Ac-ox LDL | Suzuki et al., 1997; Kunjathoor et al., 2002 |
| | β-amyloid fibrils | El Khoury et al., 1996 |
| | Advanced glycation end products | Araki et al., 1995 |
| | Lipopolysaccharide | Hampton et al., 1991 |
| | Lipoteichoic acid | Dunne et al., 1994 |
| | Malondialdehyde-acetaldehyde-serum albumin | Duryee et al., 2004 |
| SR-B1/SCARB1 | Unmodified LDL | Kozarsky et al., 1997 |
| | Oxidized LDL | Kozarsky et al., 1997 |
| | VLDL | Kozarsky et al., 1997 |
| | HDL | Acton et al., 1996; Kozarsky et al., 1997; Varban et al., 1998 |
| | Vitamin E | Reboul et al., 2006 |
| | Carotenoids | During et al., 2005 |
| | Silica | Tsugita et al., 2017 |
| CD36 | HDL | Brundert et al., 2011 |
| | LDL | Calvo et al., 1998 |
| | VLDL | Calvo et al., 1998 |
| | Anionic phospholipids | Rigotti et al., 1995 |
| | Apoptotic bodies | Savill et al., 1991 |
| | Collagen | Tandon et al., 1989a |
| | Aldehyde modified proteins | Duryee et al., 2005 |
| SR-E1/LOX-1 | Oxidized LDL | Chen et al., 2001b |
| | Apoptotic bodies | Li and Mehta, 2000 |
| | C-reactive protein | Shih et al., 2009 |
| | Bacteria | Chen et al., 2001a |
| | Platelets | Oka et al., 1998 |
| | Anionic phospholipids | Oka et al., 1998 |
| | MAA-Alb | Duryee et al., 2005 |
| SR-H1/STABILIN-1 | SPARC | Kzhyshkowska et al., 2006b |
| | Heparin | Pempe et al., 2012 |
| | oxLDL | Li et al., 2011 |
| | Advanced glycation end-products | Li R. et al., 2009 |
| | Phosphatidylserine | Park et al., 2009 |
| | Phosphorothioate antisense oligonucleotides | Miller et al., 2016 |
| | Placental lactogen | Kzhyshkowska et al., 2008 |
| | GDF-15 | Schledzewski et al., 2011 |
| SR-H2/STABILIN 2/HARE | Hyaluronan | Yannariello-Brown et al., 1997 |
| | PINP | McCourt et al., 1999 |
| | Heparin | Harris et al., 2008 |
| | Chondroitin sulfates A-E | Harris et al., 2004; Harris and Weigel, 2008 |
| | oxLDL | Li et al., 2011 |
| | Phosphorothioate antisense oligonucleotides | Miller et al., 2016 |
| | Phosphatidylserine | Park et al., 2008 |
| | Advanced glycation end-products | Li R. et al., 2009; Qian et al., 2009 |
| | VWF-FVIII | Swystun et al., 2018 |
| | Acetylated LDL | Harris and Weigel, 2008 |
| | GDF-15 | Schledzewski et al., 2011 |
| SR-E3/MANNOSE RECEPTOR/(CD206) | GalNAc-4-sulfate | Fiete et al., 1998 |
| | Chondroitin sulfates A and B | Fiete et al., 1998 |
| | Terminal mannose | Ezekowitz et al., 1990 |
| | Terminal L-fucose | Taylor et al., 1992 |
| | Terminal GlcNAc | Ezekowitz et al., 1990 |
| | Lysosomal hydrolases | Stahl et al., 1976; Elvevold et al., 2008a |

*(Continued)*

**TABLE 1** | Continued

| Receptor | Ligands | References |
|---|---|---|
| | Tissue plasminogen activator | Smedsrod et al., 1988a |
| | Procollagen C-terminal propeptides | Smedsrod et al., 1990 |
| | Collagen alpha chains/denatured collagen | Napper et al., 2006; Malovic et al., 2007 |
| | Bacterial and yeast pathogens | Stahl and Ezekowitz, 1998; Gordon, 2002; Allavena et al., 2004 |
| | Influenza, herpes simplex, HIV | Milone and Fitzgerald-Bocarsly, 1998; Reading et al., 2000; Turville et al., 2002 |
| SR-L/LRP-1 | ApoE | Hussain et al., 1999 |
| | Tissue plasminogen activator | Salama et al., 2019 |
| | Receptor associated protein (RAP) | Prasad et al., 2016 |
| | α2M, lactoferrin, factor VIII, etc. | Herz and Strickland, 2001 |
| LSECTIN/CLEC4G | Mannose oligosaccharides | Feinberg et al., 2001 |
| | Terminal GlcNAc, mannose, fucose | Liu et al., 2004 |
| L-SIGN/CD299/CLEC4M | HIV | Boily-Larouche et al., 2012 |
| | SARS-CoV | Jeffers et al., 2004 |
| | HCV | Gardner et al., 2003 |
| | vWF-FVIII | Swystun et al., 2019 |
| LYVE-1 | Hyaluronan | Banerji et al., 1999 |

## RECENTLY ESTABLISHED NOMENCLATURE FOR SCAVENGER RECEPTORS

Scavenger receptors are defined as protein receptors that bind to a broad range of ligands from exogenous sources (bacteria, yeast, viruses) and modified endogenous sources [oxidized lipoprotein, advanced glycation end (AGE) products, etc.]. In 2017, a workshop was organized by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health in the United States to develop a clear definition of the various groups of scavenger receptors known to date. This group categorized the scavenger receptors in 11 different classes (A through L) depending on their structure and function and clarified the nomenclature of the various receptors (PrabhuDas et al., 2017). This review will only discuss the role and function of scavenger receptors in the liver sinusoids. LSECs are known to express SR-A, two SR-B variants (SR-B1 and SR-B2/CD36), mannose receptor/CD206/SR-E3, SR-H1 and SR-H2, FcγRIIb and others under normal conditions (**Table 1** and **Figure 2**). LSECs are also known to express SR-E1 under various pathological conditions. Here, an overview of LSEC scavenger receptors are presented.

### Scavenger Receptor A (SR-A)

The class A scavenger receptors (also known as MSR1, SR-AI, SCARA1) were first cloned by the Kreiger group (Kodama et al., 1990; Rohrer et al., 1990) and may have been involved with binding to modified low-density lipoproteins. Scavenger receptor-A (SR-A) is a type II trimeric integral plasma membrane receptor that is encoded on chromosome 8 in both humans and mice. It is characterized by the following six domains: a transmembrane region, a spacer region, a coiled-coil region, a collagenous stretch of repeated Gly-X-P/K, an N-terminal cytoplasmic tail, and a carboxyl terminal-type specific-domain (**Figure 2**). Alternative splicing results in three variants of SR-A, namely SR-A1.1 and SR-A1.2 (Emi et al., 1993). The structure of the three isoforms are very similar and the differences lie in their cysteine-rich carboxy terminal domain which is involved in cell adhesion and, possibly, bacterial binding (Bowdish and Gordon, 2009; Yap et al., 2015). SR-A type-I is characterized with a cysteine rich carboxy terminal domain whereas SR-A type-II has short C-terminal domain lacking cysteine-rich site at the C-terminus (Rohrer et al., 1990). SR-A type-III has a truncated cysteine-rich domain (Gough et al., 1999). SR-A type1/1.1 are involved in the binding of diverse macromolecules, such as acetylated and oxidized low density lipoprotein (ac/oxLDL) (Suzuki et al., 1997; Kunjathoor et al., 2002), β-amyloid fibrils (El Khoury et al., 1996), AGE products (Araki et al., 1995), molecules present on the surface of gram negative and positive bacteria, such as lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Dunne et al., 1994). Rohrer et al. (1990) demonstrated that the fibrous coiled-coil and collagen-like domains are responsible for binding different ligands in SR-A type-1/1.1 as removal of cysteine-rich C-terminal domain in SR-A type-II did not affect its binding capacity. SR-A type-1.2 does not bind to SR-A type-1/1.1 ligands but it has been shown to negatively regulate the functions of SR-A type-1/1.1 *in vitro* (Gough et al., 1999).

SR-A expression was once thought to be only expressed in macrophages/KCs, but recent work have shown their expression in brain microglia and astrocytes, LSECs and vascular smooth muscle cells (Nagelkerke et al., 1983; Hughes et al., 1995; Christie et al., 1996; Gough et al., 1999; Godoy et al., 2012). Work conducted in rats have shown that SR-A expressed on LSECs are primarily responsible for carrying out the uptake of the artificial ligand, acLDL, in liver (Nagelkerke et al., 1983). They also theorized the role of an acLDL receptor present on LSECs in the prevention of accumulation of cholesterol under normal condition, though the pathways involved remain elusive. Similarly, another report has demonstrated the uptake

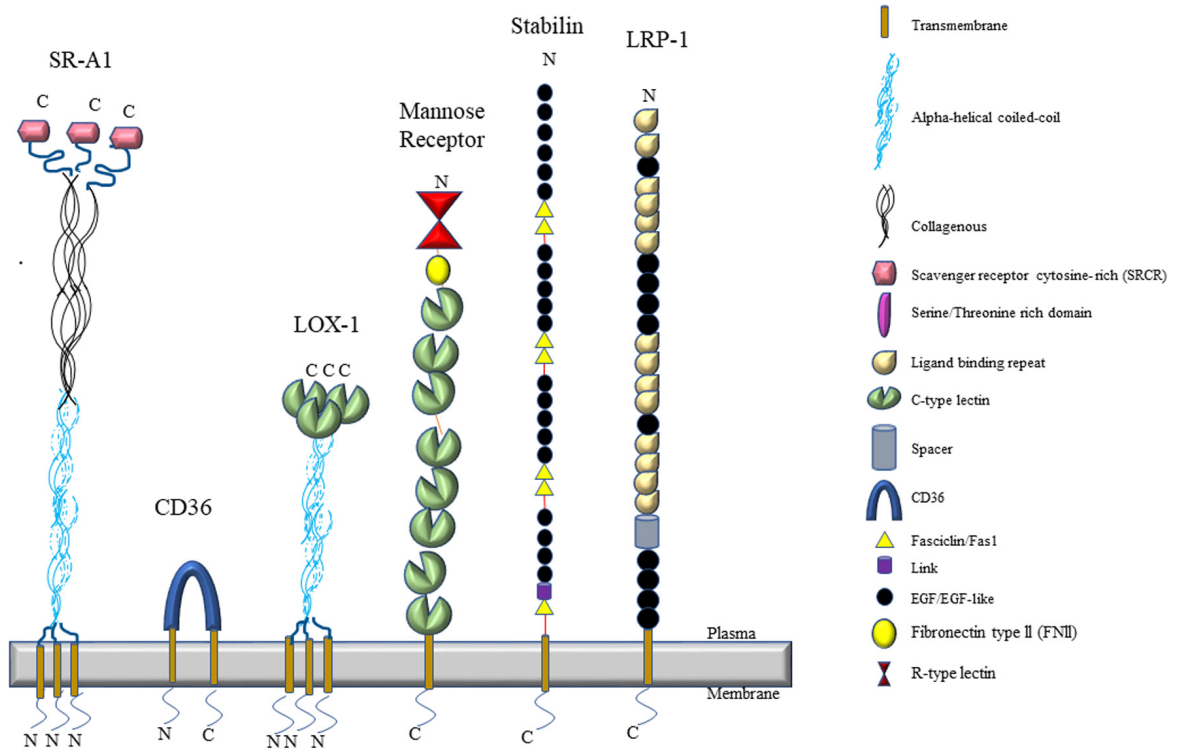


**FIGURE 2 |** An illustration of the topological features of scavenger receptors expressed by LSECs.

of ac/oxLDL by two scavenger receptors; an unidentified 95 kDa protein and the likely candidates, SR-A1/1.1 present on rat LSECs and KCs (De Rijke et al., 1992, 1994; De Rijke and Van Berkel, 1994). In 1990, a commentary in Nature written by Brown and Goldstein had attempted to bring about a universal idea or theory involving the numerous ligands including LDLs that bind to a few scavenger receptors, of which SR-A was a very likely candidate (Brown and Goldstein, 1990). Furthermore, several studies have shown the elevated expression of SR-A in atherosclerotic lesions (Matsumoto et al., 1990) and accumulation of oxLDL, a SR-A ligand in plaques, suggesting it's putative role in atherogenesis (Hiltunen et al., 2001). SR-AI in LSEC is also involved in uptake and degradation of malondialdehydes-acetaldehyde-serum albumin (MAA), which is considered immunogenic and capable of producing inflammatory responses in the liver (Duryee et al., 2005). It has also been observed that chronic ethanol administration diminishes the uptake of MAA by SR-A in rats. This leads to the accumulation of acetaldehyde and aldehyde modified protein adducts in the circulation. These adducts cause secretion of monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-2 via hepatic stellate cells, contributing to alcoholic liver disease (Kharbanda et al., 2001).

Despite all of the evidence that SR-A receptors were involved with the uptake and catabolism of LDLs, the use of knock-out mice in the late 1990's demonstrated that the SR-A receptors had a negligible effect on liver uptake and decay of serum acLDL (Ling et al., 1997; Van Berkel et al., 1998). Furthermore, SR-A1/1.1-deficient and wild type (WT) mice were compared with the liver sequestration of other non-parenchymal ligands, namely AGE, N-terminal propeptide of type III procollagen (PIIINP) and formaldehyde-treated serum albumin. The results indicated that the SR-A receptors were of minor importance for plasma clearance of these ligands and the distribution in other tissues and organs was not altered (Hansen et al., 2002).

## Scavenger Receptor B (SR-B)

Scavenger receptor class B type 1 (SR-B1/SCARB1) is a member of scavenger receptor B, which is located on chromosome 12 in humans and chromosome 5 in mice (Acton et al., 1994). SR-B1 is a membrane glycoprotein consisting of two short cytoplasmic N- and C-terminal domains, two short hydrophobic membrane regions, and a highly N-glycosylated large looped extracellular domain (Krieger, 1999). They exist as two isoforms, SR-B1 and-B1.1, resulting from alternative splicing (Webb et al., 1997). CD36 and LIMPII Analogous (CLA-1) is the human homolog of SR-B1 (Calvo and Vega, 1993). Liver and steroidogenic tissues (adrenal glands, ovaries, and testis) have been shown to highly express SR-B1 (Acton et al., 1996). Malerod et al. (2002) were the first group to demonstrate the expression of SR-B1 in LSECs from isolated rat liver. Several year later, Ganesan et al. (2016) showed the abundant expression of SR-B1 in mouse LSECs by confocal microscopy. Mutational and knock-out studies have suggested an important role of the SR-B1 receptor in facilitating

high density lipoprotein (HDL) uptake in liver and steroidogenic tissues, thereby, its role in atherosclerosis regulation (Acton et al., 1996; Kozarsky et al., 1997; Varban et al., 1998). Other than HDL, SR-B1 also binds to oxLDL, apoptotic cells, unmodified LDL, VLDL (Kozarsky et al., 1997) as well as vitamin E (Reboul et al., 2006), carotenoids (During et al., 2005), and silica (Tsugita et al., 2017). The liver is the primary organ involved in LPS clearance and LSECs are a major contributor for this activity. SR-B1 may be involved with some of the LPS clearance, but as of this date, that is not very clear. There may be a multitude of receptors or "built-in redundancy" in this LPS clearance system so that LPS levels from the gut never get high enough to cause acute inflammation (Ganesan et al., 2016).

CD36 is another member of the Scavenger Receptor B family, which is located on chromosome 7 and chromosome 5 in humans and mice, respectively (Cao et al., 1997). The entire extracellular domain of CD36 and SR-BI share high sequence homology, though the difference lies in their transmembrane and cytoplasmic domain sequences (Acton et al., 1996). It is expressed on adipocytes, capillary endothelial cells, heart and skeletal muscles, and platelets (Talle et al., 1983; Tandon et al., 1989b; Abumrad et al., 1993; Greenwalt et al., 1995; Febbraio et al., 2001) and abundantly expressed on LSECs in the liver (Strauss et al., 2017). The role of CD36 in the metabolism of lipoprotein is well-documented (Febbraio and Silverstein, 2007; Jay and Hamilton, 2018). One study demonstrated the role of CD36 in the uptake of HDL by hepatic NPCs, and their CD36 knock-out mouse model resulted in a modest but significant decrease in the HDL uptake in both hepatocytes and NPCs (Brundert et al., 2011). Similar to SR-B1, CD36 also binds to LDL, VLDL (Calvo et al., 1998), apoptotic cells (Savill et al., 1991) as well as anionic phospholipids (Rigotti et al., 1995), and collagen (Tandon et al., 1989a). Aldehyde modified proteins are likely taken up by CD36 as the SR-A KO mice had significant, but decreased uptake of these proteins (Duryee et al., 2005). An earlier study showed the involvement of CD36 in the endocytosis and degradation of AGE products, implicating its role in diabetes (Ohgami et al., 2001). However, Nakajou et al. (2005) demonstrated that CD36 is not involved in the endocytosis of AGE proteins in LSECs, suggesting the involvement of other scavenger receptors. This is quite plausible since "AGE" is a catch-all for all glycation modifications that occur involving reducing sugars and independent laboratories likely make and use their own "AGE modified protein" preparations.

Upregulation of SR-B1 expression has been documented in the livers of mice fed with a high-fat diet as compared to control mice, suggesting crucial involvement of SR-B1 in NAFLD pathogenesis (Qiu et al., 2013). Translating that to humans, a similar higher SR-B1 expression was observed in type 2 diabetic patients, but no change in hepatic SR-B1 was observed in NASH and hypercholesterolemia patients, predicting only a minimal role of SR-B1 in NAFLD (Rein-Fischboeck et al., 2015). Patients manifesting severe alcoholic hepatitis, inflammation and oxidative stress symptoms had a correlation with a decrease of circulating paroxanase/arylesterase 1 (PON1) in the blood and stimulated alternatively activated (M2) macrophages through activation of CD36. The idea here is that activated macrophages internalize increased amounts of oxLDLs via CD36 and SRA1 and that contributes to the pathophysiology of severe alcoholic hepatitis (Maras et al., 2019). However, none of the studies have documented the contribution of SR-B1 or CD36 expressed on LSECs in maintaining a homeostatic environment in the liver or a modulation in their expression in various diseases.

## Scavenger Receptor E (SR-E)

SR-E1/LOX-1, also known as lectin-type oxidized LDL receptor or oxidized LDL receptor 1 (OLR1) is a type II transmembrane protein belonging to scavenger receptor E family. It is located on chromosome 12 in humans and chromosome 6 in mice. It consists of a transmembrane domain, a short cytoplasmic domain at the N-terminus, a connecting neck domain and a C-type lectin-like extracellular domain at the C-terminus (Sawamura et al., 1997; Park et al., 2005). Maturation of Lox-1 requires post-translational N-linked glycosylation of the extracellular domain at the C-terminus and this modification was shown to be important for binding of various ligands, intracellular transport, signaling processes, and several other biological functions (Kataoka et al., 2000). Mutational studies have shown the importance of conserved C-terminal residues in the lectin like domain in LOX-1 for ligand binding (Chen et al., 2001b). LOX-1 is expressed by endothelial cells, macrophages, vascular smooth muscle cells, adipocytes and chondrocytes in low levels (Sawamura et al., 1997; Yoshida et al., 1998; Chui et al., 2005; Akagi et al., 2006). However, an increase in expression is seen by various pro-inflammatory markers, shear stress and mechanical stimuli, such as tumor necrosis factor-alpha (TNF-α), and phorbol 12-myristate 13-acetate (PMA) *in vitro* (Kume et al., 1998; Murase et al., 1998). Despite having no homology with other oxLDL binding SRs, such as SR-A and B, it is considered as the major endothelial SR for binding, uptake, and degradation of oxLDL (Sawamura et al., 1997). Other than oxLDL, LOX-1 is also involved in the binding with apoptotic and aged cells, C-reactive protein (CRP), bacteria, platelets and anionic phospholipids, such as phosphatidylserine and phosphatidylinositol, suggesting their role in various physiological conditions (Oka et al., 1998; Li and Mehta, 2000; Chen et al., 2001a; Shih et al., 2009).

Under normal conditions, LOX-1 is expressed in low amounts, in contrast to its high expression during various pathophysiological events (Ogura et al., 2009; Balzan and Lubrano, 2018). A study has documented an increased expression of LOX-1 in the aorta of hypercholesterolemic, hyperlipidemic rabbits suggesting that a Western diet may induce this receptor for the overall metabolism of lipids (Chen H. et al., 2000; Chen M. et al., 2000). Another study was conducted on a hypertensive rat model using Dahl salt-sensitive and Dahl salt-resistant rats. Dahl salt sensitive rats develop hypertension when fed with a high salt diet and Dahl salt resistant rats show minor blood pressure changes on the same diet. They reported up-regulation of LOX-1 expression in hypertensive rats, thereby, suggesting its role in hypertension and a putative target for treating atherosclerosis (Nagase et al., 1997, 1998). The role of LOX-1 contributes to the pathology NAFLD resulting, in part, from LSEC dysfunction. Zhang et al. (2014) studied the effect

of human LSEC LOX-1 gene knockdown in oxLDL-induced hepatic injury. They found an increase in ROS production and enhanced p65 expression in oxLDL treated human LSECs. This effect was significantly reduced after LOX-1 siRNA treatment, suggesting the role of LOX-1 in activation of NF-kB and the ROS pathway. A reduction in the expression of eNOS was found in human LSEC culture treated with oxLDL, whereas, LOX-1 siRNA treatment resulted in an enhanced expression of eNOS in this culture system. They also reported a reduction in the number of fenestrae, diameter and porosity in ox-LDL-treated human LSEC culture and LOX-1 siRNA resulted in reversal of these effects, suggesting its role in stress related atherogenesis (Fraser et al., 1995). LSEC defenestration was found to be mediated by LOX-1 by upregulating ET-1 and caveolin-1. Although, the association between LOX-1, ET-1, and caveolin-1 remains unclear, oxidative stress generated due to ROS production suggests the role of LOX-1 in NASH and NAFLD pathological manifestations (Pasarin et al., 2012). Along with SR-A, LOX-1 was also shown to be responsible for degradation of MAA-Alb (Duryee et al., 2005). Furthermore, a study conducted on aortic endothelial cells reported a reduction in LOX-1 expression with aging and associated it with the progression of aging related diseases (Khaidakov et al., 2011).

Mannose receptor (MR)/CD206/SR-E3 is another member of the scavenger receptor E belonging to the C-type lectin family. It is a type I integral membrane protein which is present on chromosome 10 in humans and chromosome 2 in mice. The mannose receptor is composed of an N-terminal extracellular region and a C-terminal intracellular region. The N-terminus is composed of three distinct domains and each domain binds to its specific ligands. First, eight consecutive C-type carbohydrate recognition domains (CRDs) that bind to terminal mannose residues, N-acetylglucosamine and L-fucose (Ezekowitz et al., 1990; Taylor et al., 1992). Second, a fibronectin type-II repeat domain that binds to collagen I-IV (Martinez-Pomares et al., 2006; Napper et al., 2006). Third, a cysteine-rich domain at the N-terminus binds to sulfated sugars, such as GalNAc-4-sulfate and chondroitin sulfates A and B (Fiete et al., 1998). Besides binding of specific ligands by their respective domains, MR is involved in the binding of numerous endogenous ligands, such as lysosomal hydrolases (Stahl et al., 1976; Elvevold et al., 2008a), tissue plasminogen activator (tPA) (Smedsrod et al., 1988a), and Procollagen type I carboxy-terminal propeptide (PICP) (Smedsrod et al., 1990). MR has been implicated in the binding of several pathogens including *Candida albicans*, *Pneumocystis carinii*, and *Leishmania donovani* by its cysteine-rich domain (Stahl and Ezekowitz, 1998; Gordon, 2002; Allavena et al., 2004). More recent studies have reported MRs binding with influenza, herpes simplex virus as well as HIV (Milone and Fitzgerald-Bocarsly, 1998; Reading et al., 2000; Turville et al., 2002) with varying affinities. The MR is expressed in most tissue macrophages, LSECs, lymph node and spleen; kidney mesangial cells, and dendritic cells subsets (Linehan et al., 1999; McGreal et al., 2004; Linehan et al., 2005).

Due to their ability to recognize and bind with carbohydrate moieties present on the surface of pathogens, MR expressed on LSECs is involved in the clearance of denatured collagen (Malovic et al., 2007). This study showed a reduction in plasma clearance of radiolabeled DebColl, a heat-denatured type-1 collagen, in a MR KO mouse model *in vivo*. LSECs isolated from MR KO mice were not able to internalize radiolabeled DebColl as efficiently as WT LSECs *in vitro*. Reduction in the endocytic capacity of LSECs for removing denatured collagen might contribute to the onset of pseudo-capillarization or fibrosis (Ala-Kokko et al., 1987). Subsequently, Elvevold et al. (2008a) reported the importance of MR in maintaining the high lysosomal degradation capacity using the MR knock-out model. They found a reduction in lysosomal enzymes in freshly isolated LSECs from MR KO mice as compared to WT. They further examined the endocytic and intracellular degradative capacity of LSECs and reported that MR KO mice took twice as long to degrade injected radiolabeled formaldehyde-treated serum albumin (FSA) as compared to WT, though endocytic capacity remained the same for both cell types. In accordance with the former, the MR KO mice contained nearly twice as much radioactivity than the WT mice 2 h-post-injection. It was concluded that the cellular uptake of SR ligand is not hampered in MR KO mice and there is only a deficiency in the amount of lysosomal enzyme content, hence, reduced degradative capacity in MR KO mice. This demonstrates that the MR is involved with the turnover and homeostasis of many intrinsic molecules within the mammalian organism.

Several studies have shown the expression of MR on LSEC is impacted by cytokines and inflammatory stimuli. Asumendi et al. (1996) demonstrated the upregulation of the LSEC MR after exogenous administration of human recombinant IL-1b in the rat *in vivo*. IL-1b is a pro-inflammatory cytokine present in acute infections. A similar upregulation was observed in the LSEC MR when IL-1b was induced with LPS in the rat endogenously (Asumendi et al., 1996). Similarly, enhanced MR expression by LSECs in culture when incubated with IL-10 or IL-4 with IL-13 (Liu Y. et al., 2013) acts contrary to IL-1b and is involved with the "type-II" activation of the immune system. Similarly, a study on mouse model of C26 colon carcinoma hepatic metastasis has shown that MR expression level and endocytic capacity increases with an increase in the production of IL-1b from LSECs. The increased production of IL-1b resulted from the binding of ICAM-1 expressed on LSECs with LFA-1 on C26 colon carcinoma cells (Arteta et al., 2010). This activity decreased secretion of IFN-$\gamma$ as well as anti-tumor cytotoxicity. This study discovered the role of MR in promoting IL-1b and ICAM-1 mediated pro-metastatic effects in the liver.

## Scavenger Receptor H (SR-H)

Stabilin (or FEEL/CLEVER/HARE) receptors are class H scavenger receptors. They are type I transmembrane proteins consisting of 20–21 EGF/EGF-like domains, seven fasciclin-1 domains, an X-linked domain, a transmembrane region, and a short cytoplasmic domain (Politz et al., 2002). This family consists of two members; Stabilin-1 and Stabilin-2. Stabilin-1 is also known as SR-H1/MS-1/FEEL-1/CLEVER-1. Stabilin-2 is also known as SR-H2/FEEL-2 and HARE, which is a shorter isoform of Stabilin-2 generated by proteolytic cleavage (Goerdt et al., 1991; Weigel, 2019). The main structural difference between

Stabilin-1 and Stabilin-2 is the presence of 20 EGF-like domains in stabilin-2 as compared to 21 domains in stabilin-1. Stabilin-1 is located on chromosome 3 and chromosome 14 in humans and mice, respectively. Stabilin-2 is located on chromosome 12 in humans and chromosome 10 in mice, respectively. Their extracellular domains share 55% similar homology, but their short intracellular domains are highly diverse, which results in differential abundance in different tissues and cells (Harris and Cabral, 2019). Sinusoidal endothelial cells in the liver, spleen, adrenal cortex and tissue macrophages express Stabilin-1 (Goerdt et al., 1991; Kzhyshkowska et al., 2006a), whereas, the expression of stabilin-2 is abundant in the sinusoids of liver, spleen, lymph node and bone marrow (Yannariello-Brown et al., 1997; Weigel and Weigel, 2003; Qian et al., 2009). Both receptors are also expressed in several other tissues throughout the body at lower levels (Falkowski et al., 2003). Stabilin-1/2 are considered the primary scavenger receptors of LSECs and are responsible for the binding, uptake, and degradation of multifarious ligands, such as hyaluronan, N-terminal pro-peptide of type I procollagen (PINP) (Yannariello-Brown et al., 1997; McCourt et al., 1999), chondroitin sulfates (Harris et al., 2004), AGE (Li R. et al., 2009), oxLDLs (Li et al., 2011), SPARC (Kzhyshkowska et al., 2006b), heparin (Harris et al., 2008), von Willebrand factor-factor VIII (Swystun et al., 2018) and synthetic phosphorothioate antisense oligonucleotides (Miller et al., 2016).

Stabilin-1, and to some extent, Stabilin-2, in LSECs, are specifically involved in the uptake of oxLDL, thus playing a role in the prevention of atherogenesis (Li et al., 2011). The Stabilin receptors are also involved in the internalization and clearance of various macromolecules that cannot be cleared by the kidney due to size limitation for some of these macromolecules. In 2011, it was reported that mice lacking both Stabilin-1 and Stabilin-2 did not live as long as their WT littermates due to mild perisinusoidal liver fibrosis and severe glomerular fibrosis. This study showed that both Stabilin-1 and -2 are essential for the normal clearance of extracellular matrix material, thus preserving the homeostasis of the liver as well as other distantly located organs (Schledzewski et al., 2011). In humans, it is not known what occurs in Stabilin allelic insufficiency or loss of function. However, in de-differentiated tissues in cases of liver cancer, loss in the expression of Stabilin-1 and -2 was seen in hepatocellular carcinoma (HCC) patients, and this loss was inversely related to patient survival. Additionally, loss of Stabilin-1 and -2 as well as CD32b was also observed around the tissues surrounding the tumor in HCC patients (Geraud et al., 2013). Conversely, another study has shown that Stabilin-1 retains its expression in the LSECs of the diseased liver and mediates transmigration of T cells, especially T regulatory cells across the endothelium in the inflamed liver. They also found increased expression of Stabilin-1 in the vessels and sinusoids lining the tumor in HCC (Shetty et al., 2011). A survey was conducted on young and old rats to delineate Stabilin expression and endocytic capacity in LSECs in response to aging. Their results found that there was an attenuation in the endocytic capacity of old rats, although the degradation capacity for both ages were similar. An increase in LSEC thickness was also observed in old age rats, which might be responsible for lower endocytic capacity (Simon-Santamaria et al., 2010). Similarly, another study had documented a decrease in Stabilin-2 receptors expression on primary LSECs in an aged rat model (Maeso-Diaz et al., 2018). From these studies, we may generally conclude that aging results in reduced expression of the Stabilin receptors and disease may alter expression and function of these receptors.

LSECs are involved in the synthesis of Factor VIII, a blood coagulation factor. This pro-coagulant is either defective or missing in Hemophilia A patients (Powell, 2009). Do et al. (1999) first identified FVIII mRNA in purified LSECs and hepatocytes from mice and in cultured LSECs by RT-PCR. They also found higher FVIII mRNA expression in LSECs quantitatively. In agreement with this finding, the transplantation of LSECs isolated from FVB/N-Tie2–GFP mice in a hemophilia A mouse model restored plasma level of FVIII (Follenzi et al., 2008). An increase in plasma FVIII correlated with the proliferation of transplanted LSEC in a Hemophilia A mice model. Two months after the LSEC transplantation, a bleeding experiment was performed to evaluate coagulation. The results showed that bleeding stopped in 15–20 min after a tail-cut which corrected this disorder. To take this one step further, an interesting attempt was made to transplant human fetal LSECs in uPA-NOG (or immunodeficient) mice and evaluate human FVIII plasma levels in mice (Fomin et al., 2013). Factor VIII levels were about half as high as a normal human plasma sample, but the amount far surpassed the non-transplanted controls indicating that LSECs produce FVIII in appreciable quantities. These findings suggest a novel role of LSECs in the treatment of hemophilia A patients suffering from FVIII deficiency. Interestingly, Stabilin-2 which is highly expressed in LSECs and in the sinusoids of spleen, also regulates the clearance rates of FVIII. Based on the data derived from genome wide association studies (GWAS), both Stabilin-2 and CLEC4M (see below) have been identified to affect vWF-FVIII levels in plasma. vWF or von Willebrand factor is normally found in tight physical association with FVIII in plasma (Lollar, 1991). Using a combination of KO mice and immunofluorescence techniques, Swystun and co-workers firmly established that Stabilin-2 and CLEC4M expressed in LSECs of mice, regulates turnover of FVIII (Swystun et al., 2018, 2019).

## Scavenger Receptor L (SR-L)

LRP-1 (Low-density lipoprotein receptor-related protein or CD91 or α2macroglobulin receptor) is a scavenger and endocytic receptor present on the cell surface belonging to the family of low-density lipoprotein receptor (Herz et al., 1988). The structure of LRP-1 is composed of five domains (i) the ligand-binding domain (ii) the O-linked sugar domain (iii) the EGF-precursor homology domain (iv) the transmembrane domain and the intracellular domain. Proteolytic cleavage of LRP-1 results in alpha subunit ligand-binding domain at the N-terminus and ß subunit consisting of other remaining domain at the C-terminus (Lillis et al., 2008). LRP-1 binds to more than 30 ligands, such as ApoE (Hussain et al., 1999), tPA (Salama et al., 2019), a receptor-associated protein (RAP) (Prasad et al., 2016), in addition to trypsin-activated α2-macroglobulin (α2M*), lactoferrin, Factor VIII and others

(for a complete list of ligands, see Herz and Strickland, 2001). The presence of different motifs at the cytoplasmic tail of LRP-1, such as two dileucine motifs, NPXY motifs, one YXXL motif has been suggested to be responsible for its high endocytic uptake (Li et al., 2000; Deane et al., 2008). LRP-1 is expressed in the liver, lung, brain, intestine, and muscles (Herz et al., 1988).

Oie et al. (2011) reported the expression of LRP-1 on LSECs with immunofluorescence. They showed that LRP-1 expressed in LSECs is responsible for partial hepatic clearance of RAP, an inhibitor of all known ligand interactions with LRP-1 and α2M*, along with other hepatic cells, suggesting the role of LSECs in lipid homeostasis. A hepatocyte-specific LRP-1 KO study showed that NAFLD disease progression is accelerated by the deficiency of LRP-1 in mice fed with a high-fat high cholesterol diet and that LSEC and KC LRP-1 were not sufficient to rescue the phenotype (Hamlin et al., 2018). Similarly, another hepatocyte-specific LRP-1 KO study has demonstrated an increase in the accumulation of lipid in primary culture of LRP-1 KO hepatocytes due to an impairment in the lysosomal degradation capacity of auto-phagolysosomes leading to cell death (Hamlin et al., 2016). These studies suggests severe impact of LRP-1 dysregulation on NAFLD progression. However, not much is known about the LSEC specific function of LRP-1 in various diseases and this needs to be elucidated.

## OTHER CELL SURFACE RECEPTORS

### FcγRIIb/CD32b

Fc gamma receptors have four major classes in mice (FcγRI-IV) and six in humans (Daeron, 1997). Fc gamma receptors IIb (FcγRIIb) binds to the monomeric immunoglobulin G (IgG) Fc domain with low affinity and inhibit the response produced by activating FcγRs. It is located on chromosome 1 in both humans and mice (Fagerberg et al., 2014; Yue et al., 2014). FcγRIIb consists of a cytoplasmic region characterized by a 13 amino acid long YSLL sequence, also known as Immunoreceptor Tyrosine based Inhibitory motif (ITIM) and an extracellular domain (Van den Herik-Oudijk et al., 1995). Alternative splicing of mRNA sequence results in two isoform namely FcγRIIb1 and FcγRIIb2. ITIM is essential for its inhibitory function (Van den Herik-Oudijk et al., 1995). When immunoglobulins on the cell surface bind and crosslink these receptors, tyrosine 309 is phosphorylated. Tatsushi et al. demonstrated that mutation of Tyr 309 to phenylalanine in the ITIM motif abolished its inhibitory effect of B cell activation (Amigorena et al., 1992; Muta et al., 1994). Daeron et al. (1993) demonstrated that a single mutation of Tyr26 to glycine at the intra-cytoplasmic domain and YSLL motif resulted in impairment of endocytosis and phagocytosis processes, suggesting that phosphorylation or dephosphorylation of tyrosine residues is important for normal function of the receptor. Similarly, another study reported that single amino acid substitution of tyrosine to alanine using alanine scanning mutagenesis methods resulted in the abolition of endocytosis (Hunziker and Fumey, 1994). This investigation discovered that the endocytosis signaling and di-leucine-based signaling overlap so that you could not have one without the other in terms of function. The expression of FcγRIIb is found on the sinusoids of spleen and liver as well as in immune cells, such as B cells, dendritic cells, myeloid cells as well as leukemia and lymphoma cells (Lim et al., 2011; Tutt et al., 2015).

FcγRIIb has been implicated in the regulation of immunoreactivity. One study has reported a decrease in expression of FcγRIIb on B cells in active systemic lupus erythematosus (SLE) patients as compared to healthy control, suggesting its protective role in SLE (Ochi and Kawabi, 1992). Similarly, an increase in FcγRIIb expression on B cells upon delivery of retroviral transduced FcγRIIb bone in spontaneous lupus-prone mice compared to the mice that received parent retrovirus transduced bone marrow (McGaha et al., 2005). They also found a decrease in the immune complex accumulation in the kidney as compared to control. Besides its expression on hematopoietic cells, FcγRIIb expression is also found exclusively on LSECs, and it is used as a marker to distinguish LSECs from other liver cell types (Mousavi et al., 2007). In hepatic sinusoids, FcγRIIb is responsible for removing small immune complexes (*SIC*). Ganesan et al. (2012) studied the blood clearance rate of *SIC* using radio iodinated *SIC* in WT and FcγRIIb KO mice model. They found an inhibition in the clearance rate of *SIC* in FcγRIIb KO mice as compared to control, suggesting the involvement of LSECs FcγRIIb in immune complex mediated diseases (Ganesan et al., 2012).

A study conducted on NAFL and NASH biopsy specimens to assess the expression of FcγRIIb on LSECs reported a medium negative correlation between serum collagen type IV and hyaluronan with FcγRIIb expression (Ishikawa et al., 2019). An increase in type IV collagen and hyaluronan contents have already been shown with NASH progression, suggesting a reduction in scavenger function of FcγRIIb. They also witnessed an inversely proportional relation between FcγRIIb expression and fibrosis stages, reporting the highest expression at the initial stage of fibrosis and lowest at fibrosis stage 3. However, taking all of the data together across the various grades of fibrosis and NAFLD activity scores, Ishikawa and coworkers did not find a significant difference in expression level of FcγRIIb. A new insight in the hepatic fibrosis study was provided by Vilar-Gomez et al. (2017) who reported platelet counts as a novel indirect biomarker for portal hypertension and advanced hepatic liver disease assessment. They reported the inhibitory role of platelets in fibrosis. In agreement with this study, Ishikawa et al. (2019) have shown a positive relationship between platelet count and FcγRIIb expression on LSECs through regression analysis, suggesting a low platelet count in high fibrosis stage, thereby, decreased FcγRIIb expression. For HCC, a decrease in FcγRIIb expression is co-commitment with an increase in cancer grade similar to Stabilin-2 (Geraud et al., 2013). A study conducted on peritumoral tissue samples of HCC patients showed that the expression of FcγRIIb was decreased in 63% of samples taken into consideration for the study. A microarray analysis of these tissues found that loss of FcγRIIb is related to significantly longer tumor-specific survival. However, in terms of the 5-yr survival rate, there

was more of an impact or survival rate for Stabilin-2 (42%) than FcγRIIb (16%).

Another ligand of FcγRIIb, fibrinogen-like protein 2 (FGL2), was found to be increased in NAFLD patients demonstrating severe forms of NAFLD, suggesting a decrease in FcγRIIb expression (Colak et al., 2011). This correlation did not hold across the fibrosis stages or grades of steatosis. Furthermore, Maeso-Diaz et al. (2018) have shown an even or slight increase in FcγRIIb expression in the comparison of young and old LSECs, however, there is a very stark reduction in Stabilin-2, eNOS, BMP-2, Lamb1, and HGF in aged LSECs suggesting that the LSECs become vulnerable to acute or chronic injury in old age.

## Toll-Like Receptors

Besides scavenger receptors, LSECs express multifarious pattern recognition receptors mostly consisting of toll-like receptors (TLRs). TLRs are capable of recognizing pathogen-associated molecular patterns (PAMP) present on invading microbes or damage-associated molecular pattern (DAMP) originating from endogenous damaged or apoptotic cells (Kawasaki and Kawai, 2014). They are type I transmembrane glycoprotein consisting of an extracellular N-terminal ligand-binding domain, single transmembrane domain, and a C-terminal cytoplasmic domain. Ligand binding is mediated through an ectodomain characterized by leucine-rich repeats (Bell et al., 2003). Downstream signaling is mediated by adaptor proteins associated with the Toll/IL-1 receptor (TIR) present at the cytoplasmic C-terminal domain (O'Neill and Bowie, 2007). TLRs form an important bridge between innate and adaptive immune response system (Werling and Jungi, 2003; Pasare and Medzhitov, 2004). TLRs also induce the production of pro-inflammatory and effector cytokines and aid in the activation of T-cells by upregulating co-stimulatory molecules present on antigen-presenting cells (Vasselon and Detmers, 2002). Humans express 10 TLRs (TLR 1–10), whereas, mice express 12 TLRs (Hopkins and Sriskandan, 2005). Of these, LSECs express seven Toll-like receptors; TLR1–4, 6, 8, 9.

Uhrig et al. (2005) demonstrated a constitutive expression of TLR4 on cultured LSECs isolated from mice. TLR4 binds to LPS present on gram-negative bacteria and initiates an immune response for its clearance (Poltorak et al., 1998). In this study, cultured LSECs developed tolerance upon repetitive exposure with LPS, though this effect was not mediated by downregulation in the expression of TLR4. Reduction in the activation of NF-kB was responsible for developing tolerance against LPS stimulation in LSECs. Not too long thereafter, Martin-Armas et al. (2006) showed that CpGs are taken up by TLR9 present in murine LSECs. The bacterial DNA is characterized by unmethylated CpG motifs that act as a potent immune stimulator by inducing the production of cytokines from various immune cells, such as dendritic cells, macrophages, B cells and NK cells (Ashkar and Rosenthal, 2002; Dalpke et al., 2006). These studies reported the presence of TLR9 in murine LSECs for the first-time using RT-PCR and immunolabeling. LSECs accumulate the most FITC-labeled CpG than other liver cell types shown by both *in vitro* and *in vivo* experiments. They also reported the binding of CpG to TLR9 in the endo-lysosomal compartment which activated NF-κB signaling for the IL-1β and IL-6 production. This study suggests the role of LSECs in mediating innate immune response in the liver. A TLR4 KO mouse study demonstrated the role of TLR4 in NAFLD by enhancing the secretion of hepatic TGF-ß and collagen associated with fibrosis (Sutter et al., 2016). These results strongly suggest that the chronic inflammation associated with fatty liver disease is regulated, in part, by TLR4.

What is the functional role of TLRs in response to ligands in LSECs? To answer this question, responses to many TLR specific agonists were evaluated in murine LSECs (Wu et al., 2010). Upon treatment with TLR specific agonists, the high mRNA expression level of TLR1,4,6,8 and moderate mRNA expression level of TLR9 were observed in murine LSEC, however, TLR5, 6, and TLR9 showed very low mRNA expression. Contrary to mRNA expression, TLR5 and TLR6 did not show any expression at the protein level in flow cytometry. They also showed that the antiviral response is produced by TLR3 in LSECs by the secretion of IFN-β. Furthermore, TNF-α production was reported in LSECs treated with TLR4 agonists in high amounts and by TLR2,3 and TLR8 in a moderate amount. Similarly, a 4-fold and 16-fold upregulation was observed in LSECs treated with TLR4 and TLR3 agonists, respectively. LSECs were also able to upregulate MHC class II expression with TLR8 agonists as well as the proliferation of T-cells with TLR1,2 and TLR6 agonists. Additionally, LSECs were involved with the activation of CD4 and CD8 T-cells when treated with TLR1,2 and TLR6 agonists. These results suggest that LSECs are capable of initiating antiviral and pro-inflammatory responses by TLR3 as well as adaptive responses through TLR1,2,6 and TLR8, thereby maintaining hepatic immune response. Similarly, the role of TLR3 stimulated the murine LSECs is reported in suppressing Hepatitis B viral replication mediated by IFN-ß production *in vitro* (Wu et al., 2007).

The portal circulation continuously exposes the liver to microbial antigens and food from the gut; therefore, it possesses a piece of special machinery to maintain immune tolerance. Liu J. et al. (2013) reported that incubation of LSECs with palmitoyl-3-cysteine-serine-lysine-4 (P3C), a TLR2 ligand, resulted in the reversal of immune tolerance. LSECs were co-cultured with stimulated T cells isolated from mice and treated with P3C. This resulted in an increase in proliferation of T cells as well as cytokine production in co-culture as compared to T cells cultured alone. An increase in CD8+ effector T cell population, IL-12 production, and decrease in PD-L1 expression on LSECs was also observed in these co-culture systems. This study outlined the role of TLRs in regulating the immunosuppressive property in LSECs. Similarly, one study has demonstrated the role of TLR2 expressed on LSECs in initiating an innate immune response toward adeno-associated viral vectors (rAAV) and efficiency of gene therapy mediated by rAAV which may be enhanced by understanding this mechanism (Hosel et al., 2012). As the anatomical sieve of the liver, the LSECs are continually monitoring antigens and confer tolerance to maintain proper homeostasis.

## L-SIGN and LSECtin

Liver/lymph node-specific ICAM-3 grabbing non-integrin (L-SIGN)/CD299L/CLEC4M and Liver and lymph node

sinusoidal endothelial cell C-type lectin (LSECtin)/CLEC4G are type II transmembrane proteins belonging to the C-type lectin family (Bashirova et al., 2001; Pohlmann et al., 2001; Gardner et al., 2003; Liu et al., 2004). These two receptors are encoded on chromosome 19 in humans (Grimwood et al., 2004). They are characterized by an intracellular domain, a transmembrane domain, and an extracellular domain composed of a neck domain and a C-type carbohydrate recognition domain (CRD). Ligand binding is mediated through C-type CRD. L-SIGN is expressed by liver and lymph node sinusoidal endothelial and placental capillary endothelial cells (Pohlmann et al., 2001). Likewise, liver and lymph node sinusoidal endothelial cells specifically co-express LSECtin (Liu et al., 2004). LSECtin expression is also seen on bone marrow sinusoidal endothelial cells as well as on KCs in the liver (Dominguez-Soto et al., 2009). L-SIGN binds to high mannose oligosaccharide (Feinberg et al., 2001), whereas, LSECtin can bind with N-acetylglucosamine, mannose, and fucose (Liu et al., 2004). Since L-SIGN is expressed on placental capillary endothelium, one study has shown their possible involvement in mother to child transmission of HIV-1 virus (Boily-Larouche et al., 2012), while several other studies relating L-SIGN with HIV-1 entry remain contradictory. L-SIGN expressed on pulmonary endothelial cells serve as the gateway for the entrance of SARS-CoV as it was able to bind HEK293T cells expressing purified soluble SARS-CoV glycoproteins (Jeffers et al., 2004). Similarly, LSECtin might also play a role in mediating SARS-CoV infection in hepatocytes (Gramberg et al., 2005).

L-SIGN has been reported to interact with glycoprotein E2 of the Hepatitis C viron (HCV) (Gardner et al., 2003). This study has shown that L-SIGN transfected Hela cells were able to bind purified HCV-E2 protein as compared to parental HeLa cells using FACS analysis. Since, mannan is a ligand for L-SIGN, incubating the recombinant L-SIGN expressing HeLa cells (HeLa-L-SIGN) with mannan inhibited binding between L-SIGN and purified HCV-E2 protein. They confirmed the finding by exposing the HeLa-L-SIGN cells to HCV-virion and detected the HCV genome in L-SIGN transfected Hela cells by RT-PCR and Southern blotting. Similarly, another group has shown the involvement of L-SIGN in facilitating the entry of HCV and passing it to nearby hepatocytes present in the liver using HCV pseudotype particles (Lozach et al., 2004). Since LSECtin and L-SIGN belongs to the same C-type lectin family and share a 32% sequence identity, Li Y. et al. (2009) demonstrated that the central domain of LSECtin binds with L-SIGN and along with the C terminal CRD domain bind with E2 glycoprotein present on HCV suggesting that LSECtin binding with L-SIGN might play a role in the HCV binding to LSECs. LSECtin is also involved in mediating T-cell immune response in the hepatic system (Tang et al., 2009). This study showed that LSECtin binds to CD44, a hyaluronan binding receptor, present on activated T cells, halting T-cell activation and proliferation, thereby preventing liver injury. Both of these receptors bind to mannose residues, which help them in clearing pathogens from circulation (Liu et al., 2004). These studies suggest that L-SIGN and LSECtin may be targeted for the treatment of HCV and inflammatory liver diseases.

## LYVE-1

Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) is a type I integral membrane glycoprotein which was firstly identified exclusively on lymph vessels responsible for sequestering hyaluronic acid in the lymph vessel endothelium (Banerji et al., 1999). Similar to its homolog CD44, LYVE-1 contains a single Link module in the extracellular domain that is responsible for Hyaluronic acid (HA) binding (Day and Prestwich, 2002). It is present on chromosome 11 in humans and chromosome 7 in mice. With the use of better antibodies, LYVE-1 expression was also detected in the liver, spleen, and lymph node sinusoidal endothelial cells in humans (Mouta Carreira et al., 2001; Akishima et al., 2004). However, a recent study conducted in rodents has revealed their expression in the non-sinusoidal endothelium of many other organs, such as lungs, heart, and adrenal gland (Zheng et al., 2016). Once regarded as the main receptor responsible for the internalization and transport of HA in the lymph circulation, a study using LYVE-1 KO mice model revealed that it is not crucial for the metabolism of HA in the lymphatic endothelium (Gale et al., 2007). Since HA is the only known ligand for LYVE-1, to date, and LSECs play a very important role in the degradation of HA, Mouta Carreira et al. (2001) hypothesized that LYVE-1 expression might be present on LSECs. They performed immunohistochemistry analysis and confirmed the expression of LYVE-1 on human and murine LSECs (Mouta Carreira et al., 2001). Since LSECs are the only LYVE-1 expressing cells in the liver, it can be used to distinguish LSEC from other liver cell types. Earlier studies had suggested a link between increased HA level and cirrhosis (Ichida et al., 1996). This study showed that LSECs from the cirrhotic and HCC liver had a lower capacity to degrade HA and serum levels of HA were increased. We now know that Stabilin-2/SR-H2/HARE previously described in this article is the determining factor for HA degradation in liver (Eriksson et al., 1983; Harris et al., 2007).

Similarly, data from murine as well as a human models of HCC reported a reduction in the LYVE-1 expression in liver tumors with immunohistochemical (IHC) analysis (Geraud et al., 2013). Additionally, a tissue microarray analysis of 191 HCC samples found complete loss of LYVE-1 expression in 83% of the cases as compared to control. There was also a positive correlation between LYVE-1 expression and histological grade of the individual tumor with a 47% loss in grade G1 (least) and 89% in grade G3 (most). Furthermore, Arimoto et al. (2010) conducted an IHC experiment on frozen normal and diseased liver tissue and found decreased expression of LYVE-1 and increased vWF expression in inflamed or fibrotic liver. A weak negative correlation was also observed between LYVE-1 expression and fibrosis stage. An ultrastructural analysis revealed loss in LSEC fenestration and appearance of the basement membrane-like structure in the diseased liver, suggesting the onset of sinusoidal capillarization. They found a similar reduction in LYVE-1 expression in chronic viral hepatitis and virus-related cirrhosis liver tissues. This study suggests the possible role of

LYVE-1 in the progression of liver fibrosis. An increase in vWF expression with a similar decrease in LYVE-1 expression poses disturbances in microcirculation. Sinusoidal capillarization results in circulatory problems and disturbs the transport of various macromolecules between blood and hepatocytes in the diseased liver state. Given their role in hepatic disease progression, LYVE-1 and vWF may be used as a potential marker for sinusoidal capillarization.

## Adhesion Molecules Expressed by LSECs

Adhesion molecules play an important role in mobilizing leukocytes at the site of inflammation. This process involves several steps and carried out by a different set of adhesion molecules, such as integrins, the selectins, and Ig superfamily members (Shetty et al., 2018). Each of the adhesion molecules are tightly regulated to maintain a homeostatic environment and their expression is modulated under certain diseased condition. Unlike capillary and microvascular endothelial cells, LSECs express few integrins. Integrins are heterodimers consisting of alpha and beta subunits mediating cell-extracellular matrix adhesion (Bokel and Brown, 2002). Couvelard et al. (1993) evaluated the expression of different cell-matrix adhesion proteins in LSECs and found that LSECs express only α1ß1 and α5ß1 under normal condition whereas, αVß3 and αIIbß3 in low and variable levels. α5ß1 acts as fibronectin receptor (Schaffner et al., 2013), whereas, α1ß1 binds to collagen (Eble et al., 1993). αVβ3 binds to vitronectin (Horton, 1997) as well as fibronectin (Van Agthoven et al., 2014) and αIIbß3 binds to fibrinogen (Wippler et al., 1994). Furthermore, they found a strong enhanced expression of α1ß1 and α5ß1 as well as αVß3 and αIIbß3 in cirrhotic liver that were faintly expressed in normal liver. In addition, other integrins (α6ß1, α6ß4, α2ß1, and α3ß1) that did not show any expression in normal liver, and showed an increased expression in cirrhotic liver, suggesting their contribution to capillarization. These integrins bind to laminin, an important component of the basal lamina of the extracellular matrix (Languino et al., 1989; Felch et al., 1992; Lee et al., 1992; Chang et al., 1995). Several studies have documented an increased deposition of collagen IV, fibronectin and laminin in LSEC basement membranes during liver fibrosis and inflammation (Walsh et al., 2000; Xu et al., 2003; Mak and Mei, 2017), suggesting a possible role of these integrins in inflammation and fibrosis.

Similar to microvascular endothelial cells, the LSEC Ig-superfamily of adhesion molecules is composed of ICAM-1 (Intercellular adhesion molecule-1), ICAM-2 (Intercellular adhesion molecule-2), VCAM-1 (Vascular cell-adhesion molecule) and PECAM-1 (Platelet endothelial cell adhesion molecule/CD31). VCAM-1 is an adhesion molecule that helps in mediating leukocyte-trans-endothelial migration. VCAM-1 expression is absent in normal liver, but is strongly enhanced under inflammatory conditions (Volpes et al., 1992). LSECs constitutively express ICAM-1 along with hepatocytes, KCs and HSCs (Gulubova, 2005; Yin et al., 2007). The expression of ICAM-1 in LSECs is found to be upregulated by several inflammatory cytokines, such as TNF-α, IL-1β, or IFN-γ (Gangopadhyay et al., 1998; Oudar et al., 1998). A study was conducted on various endothelial cells isolated from the liver after transplantation rejection (Steinhoff et al., 1993). In LSECs, weak expression of ICAM-1 and ICAM-2 was observed under normal conditions. However, an elevated expression of ICAM-1 and ICAM-2 in LSECs were observed in chronic rejection as well as sepsis or viral infection cases. VCAM-1 was also shown to be partially upregulated in LSECs of irreversible and chronic rejection conditions. This study reflected the clinical relevance of sinusoidal endothelial adhesion molecules during liver transplantation.

LFA-1 (Lymphocyte Function Associated-1) belongs to the integrin family and is present on lymphocytes and leukocytes. It is an important component in the extravasation process mediating leukocyte and lymphocyte entry into the tissues from the bloodstream (Mitroulis et al., 2015; Walling and Kim, 2018). Binding of ICAM-1 expressed on LSECs to its ligand, LFA-1, expressed in pro-inflammatory cells mediates the migration of cells across the sinusoidal lining (Wong et al., 1997). This was one of the first studies to determine that Selectins, expressed on continuous vascular endothelium, are not an essential component for leukocyte transmigration into inflamed tissue of the liver. Similarly, a co-culture study conducted with LSECs and C26 tumor cells showed that LSECs expressing ICAM-1 mediate tumor migration (Benedicto et al., 2019). They also found an increase in the inflammatory IL-1β, IL-6, TNF-α, and PGE2 in the co-culture as compare to LSECs cultured alone. Interrupting the interaction between ICAM-1 and LFA-1 expressed on LSECs and C26, respectively, reduced the secretion of observed inflammatory molecules. This suggests the role of ICAM-1 and LFA-1 interaction in providing an inflammatory microenvironment suitable for colonization of tumor cells in the liver.

Another Ig superfamily adhesion molecule is PECAM-1 (CD31) (Newman et al., 1990). It is an adhesion molecule located at the cellular side and is involved in mediating endothelial cell-cell adhesion. CD31 is also responsible for carrying out leukocyte and monocyte trans-endothelial migration (Albelda et al., 1991). Whether LSECs express CD31 or not has long been debated and remains cryptic (Couvelard et al., 1993; DeLeve et al., 2004; Elvevold et al., 2008b). An upregulation in CD31 expression has been shown during the capillarization of LSECs in cirrhotic human liver (Couvelard et al., 1993). Also, its enhanced expression is detected in LSECs during focal nodular hyperplasia (Scoazec et al., 1995). Likewise, enhanced expression of CD31 has been related to the non-fenestrated and de-differentiated state of LSECs (DeLeve et al., 2006). Neubauer et al. (2000b) for the first time showed the expression of CD31 on LSECs and found no observable difference in CD31 expression under normal conditions and carbon tetrachloride-induced ($CCl_4$) liver fibrosis. However, another study by the same authors demonstrated a constitutive expression of CD31 on LSECs, whereas, a decrease of CD31 was observed after $CCl_4$ administration mediated by TNF-α *in vitro*. They hypothesized that reduced CD31 expression might aid in more desirable

mononuclear cell transmigration (Neubauer et al., 2000a). Clearly, the on-going debate for the expression of CD31 on LSECs needs to be resolved and it should never be used as a marker for rodent LSEC purification.

## PERSPECTIVES AND CONCLUSION

The past 35 years have proven to be a treasure-trove of discovery of the unique sinusoidal endothelium in liver. Advancements in their purification from rats and mice have enabled researchers to assess their physiological and biological role in normal and diseased phenotypes. Starting with the efforts of Seglen (1976) in the early 1970s and culminating to the current date, the procedure of dissociating the liver with collagenase-based enzyme mixtures have been very similar. The final steps in purification have involved differential centrifugation, fluorescent-activated cell sorting, and magnetic-activated cell sorting or a combination of these methods. A recent review on these techniques may be found in Meyer et al. (2016).

The anatomical shape and position of LSECs make them uniquely optimal for sequestering macromolecular materials from the blood. In this position, they act as "guardians" of the liver expressing numerous scavenger receptors and constantly monitoring the antigenic profile of the blood. As the liver is bathed in portal vein blood which drains the GI tract, there are many food and bacterial antigens flowing through in which LSECs play a major role in cleaning up and tempering other immune cells within the liver. **Table 1** of this manuscript outlined many of the exogenous and endogenous ligands for all of these receptors. Redundancy in binding of multiple ligands shared by several receptors suggest the importance of physiological homeostasis with regards to external material coming into contact with blood and internal tissues. What is not known so much is the biochemistry of how these receptors are taking up multiple ligands at the same time or which amino acids/domains are interacting with each ligand. We think that this is an important step forward with the advancements in crystallography and cryo-EM methodologies. With an understanding of receptor-ligand interactions, pharmacological agents may be made to enhance or block these interactions according to required circumstances in the patient.

## AUTHOR CONTRIBUTIONS

EP wrote the manuscript. AN wrote part of the manuscript and created the figures. EH edited and organized the manuscript with some brief writing in various sections. All authors contributed to the article and approved the submitted version.

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REVIEW
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# The Role of Sinusoidal Endothelial Cells in the Axis of Inflammation and Cancer Within the Liver

*Alex L. Wilkinson†, Maria Qurashi† and Shishir Shetty**

*Centre for Liver and Gastrointestinal Research, Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom*

Liver sinusoidal endothelial cells (LSEC) form a unique barrier between the liver sinusoids and the underlying parenchyma, and thus play a crucial role in maintaining metabolic and immune homeostasis, as well as actively contributing to disease pathophysiology. Whilst their endocytic and scavenging function is integral for nutrient exchange and clearance of waste products, their capillarisation and dysfunction precedes fibrogenesis. Furthermore, their ability to promote immune tolerance and recruit distinct immunosuppressive leukocyte subsets can allow persistence of chronic viral infections and facilitate tumour development. In this review, we present the immunological and barrier functions of LSEC, along with their role in orchestrating fibrotic processes which precede tumourigenesis. We also summarise the role of LSEC in modulating the tumour microenvironment, and promoting development of a pre-metastatic niche, which can drive formation of secondary liver tumours. Finally, we summarise closely inter-linked disease pathways which collectively perpetuate pathogenesis, highlighting LSEC as novel targets for therapeutic intervention.

**Keywords: liver sinusoidal endothelial cell, capillarisation, endothelial dysfunction, inflammation, leukocyte recruitment, fibrosis, hepatocellular carcinoma, metastasis**

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*Wing-Kin Syn, Medical University of South Carolina, United States*

***Correspondence:**
*Shishir Shetty*
*S.Shetty@bham.ac.uk*

*† These authors have contributed equally to this work*

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## INTRODUCTION

Globally, liver disease is estimated to cause around two million deaths per year, and together, cirrhosis and liver cancer account for 3.5% of all deaths worldwide (Rowe, 2017; Asrani et al., 2019). As the fifth most common cancer and the second leading cause of all cancer-related deaths, hepatocellular carcinoma (HCC) is becoming increasingly prevalent, and is associated with a significant global health burden (Degasperi and Colombo, 2016; Bertuccio et al., 2017; Global Burden of Disease Cancer Collaboration et al., 2017; Asrani et al., 2019). Chronic inflammation plays a critical role in driving the development of HCC, as 90% of patients have an underlying chronic liver disease (CLD) (O'Rourke et al., 2018). Moreover, the liver's permissiveness to metastasis and the hepatic tropism of many solid cancers makes the liver a frequent site of secondary tumour deposits (Budczies et al., 2015; Mielgo and Schmid, 2020). Since metastasis is responsible for up to 90% of all cancer-related deaths (Chaffer and Weinberg, 2011), better understanding of the factors which permit growth of secondary malignancies is urgently needed. In this review, we will discuss how the unique phenotype and function of liver sinusoidal endothelial cells (LSEC) can contribute in diverse ways to the development of inflammation-induced primary cancer and secondary tumours within the liver.

Liver disease follows a common pathway of progression, from inflammation to fibrosis and cirrhosis, independently of aetiology (Schuppan and Afdhal, 2008; Pellicoro et al., 2014; Koyama and Brenner, 2017). Initial liver injury, which can be toxin-induced, viral, metabolic or autoimmune in origin, causes inflammation which if unresolved can result in chronic hepatitis. Damage to the hepatocytes and changes in the liver microenvironment lead to fibrogenesis (aberrant wound healing and extracellular matrix (ECM) deposition), which can distort the liver architecture and impair liver function and regeneration (cirrhosis). Around 80–90% of HCC cases arise on a background of cirrhosis (Davis et al., 2008; O'Rourke et al., 2018) and, as such, risk factors for HCC development include viral hepatitis, alcoholism and obesity (Degasperi and Colombo, 2016; British Liver Trust, 2019). Many patients are asymptomatic until their liver disease presents at an advanced stage, due to the remarkable compensatory capacity of the liver to fulfill its function even after suffering extensive damage (Schuppan and Afdhal, 2008). Patients with advanced disease have limited therapeutic options and the best outcomes are seen in those patients who are able to undergo liver transplantation. Yet, only 10% of the global transplant demands are fulfilled by current rates (Asrani et al., 2019), thus, there is a vast unmet clinical need to develop novel treatments for liver disease patients.

The liver is strategically positioned to carry out its metabolic and immunological function, since it receives 70–80% of its blood supply from the gastrointestinal tract via the hepatic portal vein, and the remainder from the hepatic artery (Mathew and Venkatesh, 2018). The liver is exposed to countless microbial- and food-derived antigens within the capillary beds of the liver, referred to as the hepatic sinusoids. These vascular beds are lined with specialised discontinuous endothelial cells, known as LSEC, which not only form a unique barrier between the bloodstream and the parenchyma, but also play an integral role in liver physiology, immunology and pathophysiology (Shetty et al., 2018).

An emerging role for LSEC in the development and progression of both CLD and HCC has become evident over the past few decades (Elvevold et al., 2008b; Sorensen et al., 2015; Marrone et al., 2016; DeLeve and Maretti-Mira, 2017; Natarajan et al., 2017; Poisson et al., 2017; Shetty et al., 2018; Hammoutene and Rautou, 2019). Furthermore, the frequent growth of secondary tumours within the liver often requires interactions with LSEC, contributing to a metastatic niche which is exploited by numerous other cancer types (Mielgo and Schmid, 2020). We discuss the role of LSEC in disease pathogenesis and tumour development, highlighting the potential for these cells to be targeted in novel therapeutic approaches.

# LSEC PHYSIOLOGY

## Structure and Location

LSEC are the most abundant non-parenchymal cell type in the liver, representing approximately 15–20% of liver cells but only 3% of the total liver volume (Blouin et al., 1977; Poisson et al., 2017; Shetty et al., 2018). Lying at the interface between the systemic arterial and portal venous blood within the sinusoids and the liver parenchyma, they form a unique barrier between the circulation, and the underlying hepatocytes and hepatic stellate cells (HSC) within the space of Disse (Sorensen et al., 2015; Shetty et al., 2018). LSEC are highly specialised in that they have minimal basement membrane and fenestrations, arranged in sieve plates, rendering LSEC the most permeable endothelial cells in the mammalian body (Braet and Wisse, 2002; Poisson et al., 2017). Furthermore, LSEC are ideally positioned to process and recycle blood-borne proteins and lipids both from the gastrointestinal tract and the systemic circulation, thus representing the most powerful scavenger system in the body (Shetty et al., 2018). This is supported by the plethora of endocytic and scavenger receptors expressed in LSEC (Sorensen et al., 2015). Moreover, the atypical cell junctions between LSEC and the low shear environment within the hepatic sinusoids results in differences in leukocyte trafficking compared to the conventional leukocyte adhesion cascade, which may yield specific targets for recruitment during liver disease (Shetty et al., 2008; Patten et al., 2019). It is these key features which distinguish LSEC from other endothelia, allowing them to carry out their homeostatic, filtration, endocytic (**Figure 1**) and immunological functions (**Figure 2**).

## Regulation of Hepatic Blood Flow

The liver sinusoids are characterised by low shear flow compared to other capillary beds to maximise time for fluid and solute exchange to occur (Poisson et al., 2017; Mathew and Venkatesh, 2018). LSEC are key regulators of hepatic vascular blood pressure via production of vasodilatory mediators in response to shear stress (**Figure 1**). This effect is mediated by activation of transcription factor Krüppel-like factor 2 (KFL2), resulting in release of nitric oxide (NO), via endothelial nitric oxide synthase (eNOS) activity in LSEC (Shah et al., 1997; Rockey and Chung, 1998; Parmar et al., 2006; Gracia-Sancho et al., 2011). Simultaneously, shear stress downregulates expression of vasoconstrictive factors, such as endothelin-1 (ET-1), via KLF2 activation (Parmar et al., 2006). These molecules act in a paracrine manner on HSC within the space of Disse, maintaining their quiescent state and thus inhibiting their vasoconstrictive effects (Kawada et al., 1993; Deleve et al., 2008).

Whilst there is some suggestion that LSEC themselves may mediate vascular flow by swelling to form inlet and outlet sphincters (McCuskey, 1966, 2000), the most accepted concept is that LSEC regulate blood flow indirectly via HSC (Rockey, 1997). Hepatic stellate cells are contractile cells, expressing smooth muscle proteins desmin and α smooth muscle actin (αSMA), which enwrap the sinusoids and are ideally positioned to regulate hepatic blood flow (Kawada et al., 1993; Rockey, 1997). As such, LSEC remain in close proximity to HSC via interactions between CXCR4 and CXCL12/stromal-derived factor 1α (SDF1α) released by HSC, along with platelet-derived growth factor β (PDGFβ)-PDGFRβ interactions. Alongside keeping LSEC and HSC in close contact, this cell-cell communication is crucial

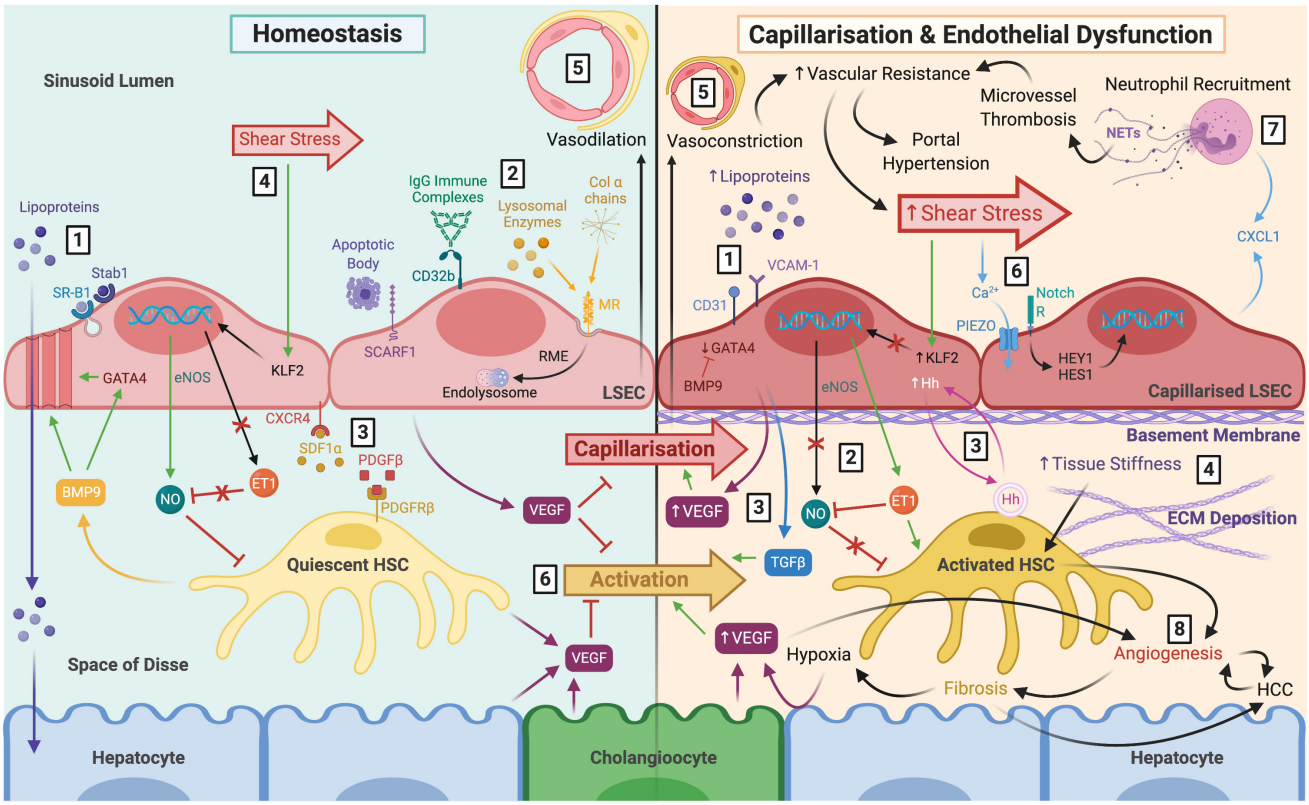


**FIGURE 1 |** The role of LSEC in maintaining homeostasis and disease pathology following capillarisation and endothelial dysfunction. *Left:* LSEC have a distinct morphology which facilitates their homeostatic function. **(1)** Lack of basement membrane and fenestrations arranged in sieve plates permit relatively free movement of macromolecules, such as lipoproteins, towards hepatocytes within the parenchyma. Lipoproteins can also be endocytosed by scavenger receptors SR-B1 and Stab1. **(2)** Scavenger receptors also facilitate uptake and clearance of waste products including apoptotic cell bodies (SCARF1), IgG immune complexes (CD32b), lysosomal enzymes (MR), and collagen α chains (MR). **(3)** LSEC remain in close proximity with HSC within the space of Disse via CXCR4-SDF1α and PDGF-β PDGFR-β interactions. **(4)** LSEC maintain HSC quiescence in response to shear stress through eNOS-dependent NO production, and inhibition of ET-1, via transcription factor KFL2. **(5)** The differentiated LSEC phenotype maintains vasodilation of the sinusoids. **(6)** VEGF production by LSEC, HSC, hepatocytes and cholangiocytes also maintain HSC quiescence and prevent LSEC capillarisation. *Right:* **(1)** Capillarisation is associated with upregulation of VCAM-1 and CD31, loss of GATA4 signalling, reduced fenestrations, and basement membrane synthesis, leading to hyperlipoproteinaemia. This can be prevented by BMP9. **(2)** Endothelial dysfunction is the inability to produce NO in response to shear stress, and paired with ET-1 synthesis, results in HSC activation. **(3)** Additional angiocrine signals release from capillarised LSEC also perpetuate HSC activation, such as excess VEGF, Hh signals and TGFβ **(4)** Activated HSC begin to deposit ECM which increases tissue stiffness, further stimulating HSC activation. **(5)** HSC respond by causing vasoconstriction which increases vascular resistance and shear stress. **(6)** It is thought that LSEC respond to these mechanocrine signals via PIEZO channels, notch-dependent HEY1 and HES1 translocation and subsequent CXCL1 secretion. **(7)** This leads to neutrophil recruitment, and NETosis induces microvessel thrombosis which perpetuates increased vascular resistance resulting in portal hypertension. **(8)** Ultimately, capillarisation and endothelial dysfunction precede angiogenesis and fibrosis, which increase the risk of cirrhosis and HCC. LSEC, liver sinusoidal endothelial cells; SR-B1, scavenger receptor class B type 1; Stab1, stabilin-1; SCARF1, scavenger receptor class F member 1; CD32b, Fcγ receptor 2b; MR, mannose receptor; RME, receptor-mediated endocytosis; HSC, hepatic stellate cell; CXCR4, C-X-C chemokine receptor type 4; SDF1α, stromal-derived factor 1α; PDGFβ platelet-derived growth factor β; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; ET-1, endothelin-1; KLF2, Krüppel-like factor 2; VEGF, vascular endothelial growth factor; VCAM-1, vascular cell adhesion molecule 1; BMP9, bone morphogenic protein 9; Hh, hedgehog; TGFβ, transforming growth factorβ ECM, extracellular matrix; NETs, neutrophil extracellular traps; HCC, hepatocellular carcinoma.

for vascular tube maturation and integrity during angiogenesis (Hellstrom et al., 1999).

## Barrier Function and Endocytic Properties

The discontinuous nature of the hepatic sinusoidal endothelia permit relatively free trafficking of macromolecules between the blood and the liver parenchyma (**Figure 1**). The fusion of luminal and abluminal plasma membrane at sites other than cell junctions form fenestrae with diameters of ∼50–150 nm which, unlike other types of fenestrated endothelia, such as those in the pancreas and adrenal glands, lack a diaphragm (Wisse et al., 1985; Stan et al., 2012). Fenestral diaphragms are comprised of plasmalemma vesicle-associated protein (PLVAP) which is the only known molecular component of these structures (Stan et al., 2004, 2012; Ioannidou et al., 2006; Herrnberger et al., 2012). It is thought that PLVAP forms homodimers arranged in radial fibrils, that are anchored to the cell membrane, which regulate vascular permeability by forming a size-selective sieve (Stan, 2004; Rantakari et al., 2015). The role of PLVAP in regulating vascular

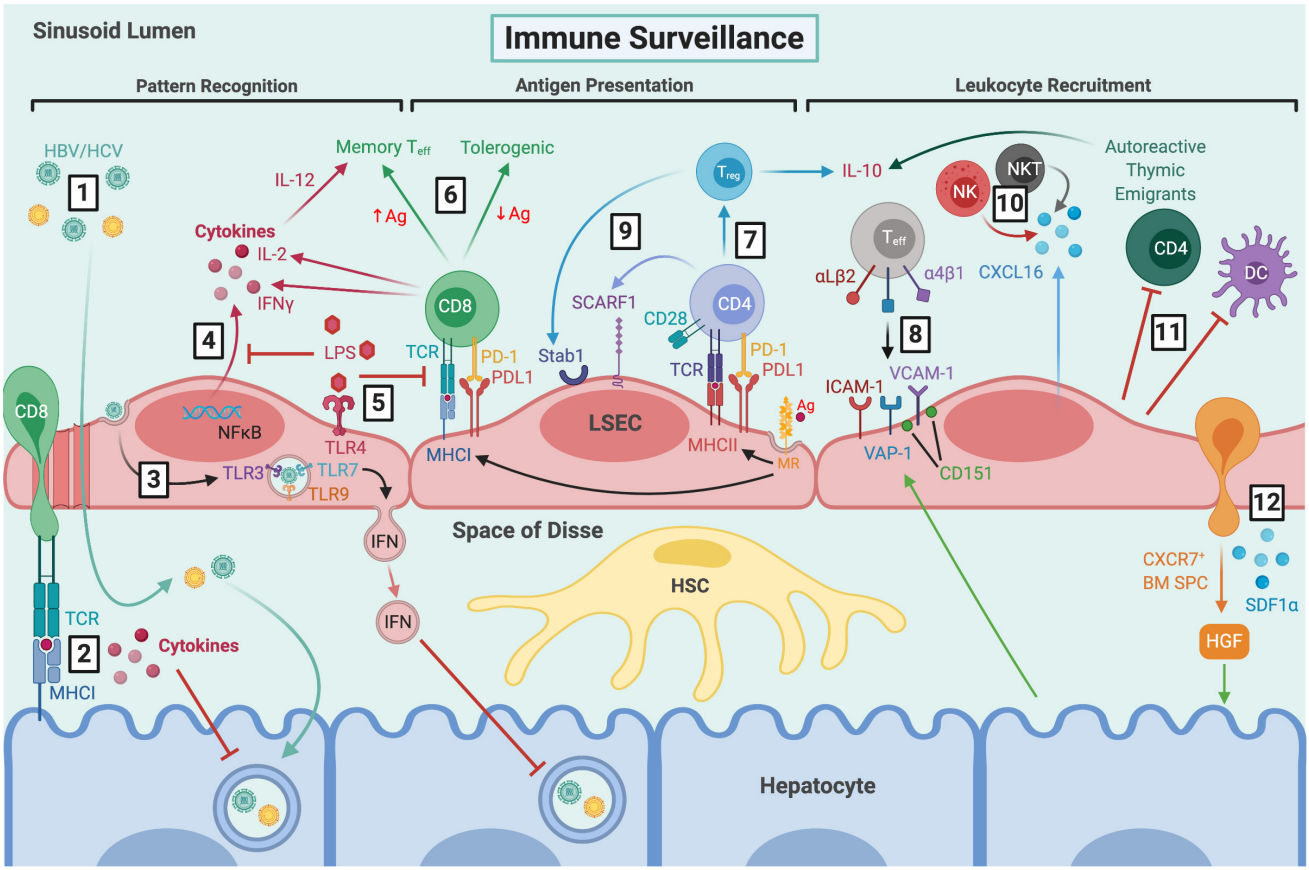


**FIGURE 2 |** LSEC maintain immune tolerance and facilitate immune surveillance by several mechanisms. **(1)** Viral particles gain access to the parenchyma through fenestrations and HBV/HCV can then go on to infect hepatocytes. **(2)** CD8$^+$ T cells extend protrusions through fenestrations and can probe for viral antigens presented by infected hepatocytes via MHCI. **(3)** LSEC can also take up viral particles via transcytosis, and RNA sensing by intracellular TLRs leads to production of IFN-rich exosomes which inhibit viral replication. **(4)** LSEC express pathogen recognition receptors, including TLR4 and NOD1/2, which signal via NFκB leading to cytokine production. **(5)** Clearance of dietary LPS via TLR4 induces tolerogenic responses by inhibiting NFκB translocation and antigen presentation. **(6)** MR-mediated antigen uptake and presentation by MHCI induces tolerogenic CD8$^+$ T cell responses in the presence of PDL1, which can be overcome by excess TCR signalling in response to high antigen concentrations. **(7)** MR-mediated uptake also precedes antigen presentation to CD4$^+$ T cells via MHCII, leading to $T_{reg}$ induction in the presence of PDL1 and absence of co-stimulation. **(8)** Typically, classic adhesion molecules VCAM-1 and ICAM-1, as well as VAP-1, are involved in $T_{eff}$ recruitment. VCAM-1 is often arranged in microdomains, forming endothelial adhesive platforms in associated with tetraspanin CD151. Hepatocytes can mediate leukocyte recruitment indirectly by modulating expression of adhesion molecules. LSEC are also involved in recruitment of distinct leukocyte subsets via atypical adhesion molecules and chemokines, such as **(9)** CD4$^+$ and $T_{reg}$ cells by SCARF1 and Stab1, respectively, and **(10)** production of CXCL16 which promotes retention of CXCR6$^+$ NK and NKT cells. **(11)** LSEC also contribute to immune tolerance by inhibiting DCs and promoting apoptosis of CD4$^+$ autoreactive thymic emigrants. **(12)** LSEC recruit CXCR7$^+$ BM SPCs via SDF1α, which mediate hepatocyte proliferation via HGF production and thus, liver regeneration. LSEC, liver sinusoidal endothelial cells; HBV, hepatitis B virus; HCV, hepatitis C virus; TCR, T cell receptor; MHCI, major histocompatibility complex class I; TLR, toll-like receptor; IFN, interferon; NOD, nucleotide-binding oligomerisation domain; NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; LPS, lipopolysaccharide; MR, mannose receptor; Ag, antigen; PD-1, programmed cell death protein 1; PDL1, programmed death ligand 1; $T_{eff}$, effector T cell; IL-2, interleukin-2; MHCII, major histocompatibility complex class II; $T_{reg}$, regulatory T cell; VCAM-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule-1; VAP-1, vascular adhesion protein 1; Stab1, stabilin-1; SCARF1, scavenger receptor class F member 1; CXCL16, C-X-C chemokine ligand 16; CXCR6, C-X-C chemokine receptor 6; NK, natural killer; NKT natural killer T; DC, dendritic cell; BM SPCs, bone marrow sinusoidal precursor cells; SDF1α, stromal-derived factor 1α; HGF, hepatocyte growth factor; HSC, hepatic stellate cell.

homeostasis has been recently reviewed (Guo et al., 2016; Bosma et al., 2018).

Despite a lack of PLVAP-containing diaphragms in adult LSEC there is evidence to suggest that PLVAP plays a critical role in development. Namely, it was recently shown that PLVAP regulates the egress of foetal liver monocyte-derived macrophages and subsequent seeding in the tissues, since these cell populations were absent in tissues from *plvap*-deficient mice (Rantakari et al., 2016). Furthermore, PLVAP forms diaphragms in foetal LSEC and is present during foetal angiogenesis in complex with lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), neuropilin-1 (NRP-1) and vascular endothelial growth factor receptor 2 (VEGFR2) (Rantakari et al., 2016; Auvinen et al., 2019). Interestingly, Auvinen et al. (2019) also demonstrated PLVAP expression in adult LSEC, which is the first report that

clearly defines the expression of PLVAP independently of fenestral diaphragms.

Early structural differentiation of the hepatic sinusoids occurs in human embryos between 5 and 12 weeks of gestation, during which time they downregulate continuous endothelial markers CD31 and CD34 and gain sinusoidal markers CD32 and intercellular adhesion molecule 1 (ICAM-1) (Couvelard et al., 1996; Poisson et al., 2017). Transcription factor GATA4 controls the distinct fenestrated phenotype which LSEC acquire by 20 weeks of gestation (Geraud et al., 2017). Under steady state conditions, fenestrated LSEC allow the passage of metabolites, plasma proteins, lipoproteins and small chylomicron remnants which are taken up by hepatocytes and HSC, whilst blood cells including erythrocytes, leukocytes and platelets are retained within the sinusoids (Poisson et al., 2017). The key role for LSEC in lipid transfer is exemplified following loss of fenestrations, where lipid uptake by hepatocytes is impaired and hyperlipoproteinaemia ensues (Clark et al., 1988; Carpenter et al., 2005; Hagberg et al., 2010; Herrnberger et al., 2014). Thus, LSEC play an integral role in fluid and nutrient exchange and metabolic homeostasis (**Figure 1**). Interestingly, fenestrations can also be observed in tumour vasculature (Hashizume et al., 2000), which may have implications for tumour persistence and progression, as well as cancer cell invasion and metastasis.

Additionally, the expression of numerous endocytic and scavenger receptors by LSEC permit their phenomenal endocytic capacity which ranks the highest of all cells in the human body (Smedsrod et al., 2009; Poisson et al., 2017). Fenestrations are dynamic structures; they are frequently associated with microtubules and actin filaments of the cytoskeleton, as well as caveolae and clathrin-coated pits, which further facilitates endocytic transport of material to and from the parenchyma (Braet et al., 2009; Mönkemöller et al., 2015). The high endocytic and lysosomal activity of LSEC means they are adept scavengers, playing an important role in the clearance of waste products from the circulation (**Figure 1**). They recognise and internalise extracellular ligands which are trafficked through the endocytic system and degraded. For instance, CD32b is the only Fcγ receptor expressed by LSEC, which mediates the clearance of small circulating IgG immune complexes (Lovdal et al., 2000; Mousavi et al., 2007).

LSEC further contribute to lipid homeostasis via endocytic uptake of high-density lipoproteins (HDLs) and oxidised or acetylated low-density lipoproteins (LDLs), which is mediated by scavenger receptor type B1 (SR-B1) and stabilin-1 (also referred to as common lymphatic endothelial and vascular endothelial receptor 1 (CLEVER-1) or fasciclin EGF-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1 (FEEL-1) and stabilin-2, respectively (Krieger, 1999; Li et al., 2011). Scavenger receptors also play a key role in maintaining glycoprotein homeostasis (Lee et al., 2002), such as clearance of advanced glycation end (AGE) products (Smedsrød et al., 1997; Svistounov and Smedsrod, 2004). One example is mannose receptor (MR), which binds numerous ligands (Martinez-Pomares et al., 2001), including collagen α chains (Malovic et al., 2007), tissue plasminogen activator (Rijken et al., 1990; Martinez-Pomares, 2012) and lysosomal enzymes which are utilised by LSEC (Elvevold et al., 2008a). LSEC are also capable of taking up antigens, via MR-mediated endocytosis and subsequent antigen presentation (Gazi and Martinez-Pomares, 2009; Martinez-Pomares, 2012; Zehner and Burgdorf, 2013), highlighting that scavenger receptors also possess important immunological functions (**Figure 2**).

# IMMUNOLOGICAL ROLE OF LSEC

## Recognition of Danger Signals and Antigen Presentation

LSEC play an integral role in both innate and adaptive immunity and contribute to maintenance of immune tolerance within the liver (**Figure 2**) (Knolle and Wohlleber, 2016; Wohlleber and Knolle, 2016). LSEC are responsible for the recognition and clearance of microbial antigens and, as such, express many pattern recognition receptors (PRRs) in addition to their scavenger receptors. LSEC respond to stimulation of toll-like receptors (TLR) 1-9 (Martin-Armas et al., 2006; Wu et al., 2010), and constitutively express three protein components of the inflammasome (Boaru et al., 2012) and intracellular nucleotide-binding oligomerisation domain (NOD)-like receptors, including NOD1 and NOD2, along with RIPK2 (Huang et al., 2018). Lipopolysaccharide (LPS) is predominantly cleared by the liver, specifically, 75% by LSEC and 25% by Kupffer cells (KC) (Mathison and Ulevitch, 1979; Yao et al., 2016). LSEC recognition of LPS is mediated by TLR4 and CD14, resulting in activation of the myeloid differentiation primary response gene 88 (MyD88) pathway, nuclear translocation of nuclear factor-κB (NF-κB), and production of pro-inflammatory mediators including interleukin-6 (IL-6) and tumour necrosis factor α (TNFα) (Hayashi et al., 2006; Wu et al., 2010; Faure-Dupuy et al., 2018). However, LSEC responsiveness to LPS diminishes following successive exposure, not due to TLR4 downregulation, but rather a reduction in NFκB nuclear translocation (Uhrig et al., 2005). This tolerogenic response reduced subsequent CD54-mediated leukocyte adhesion, and is thought to represent a mechanism by which LSEC prevent liver over-activation and inflammation in response to low-level dietary LPS (Uhrig et al., 2005). Furthermore, recognition of LPS promotes tolerance induction by downregulating antigen-presentation by LSEC and thus, T cell activation (Knolle et al., 1999).

LSEC also participate in viral clearance (**Figure 2**). It is estimated that around 90% of the viral load during an infection is cleared by LSEC, as has been shown for adenovirus and human immunodeficiency virus (HIV)-like particles, with the latter being cleared by the sinusoids at an astonishing rate of 100 million particles per minute (Ganesan et al., 2011; Mates et al., 2017). Anderson and colleagues determined the rate of clearance in mice following tail vein infusion with viral particles, by periodic sampling of peripheral blood, and analysis of viral load via quantitative PCR or ELISA over 30 min. Fluorescently labeled viral particles were localised predominantly within LSEC, as determined by fluorescent immunohistochemistry of murine livers following viral infusion. A recent study utilised real-time

deconvolution microscopy to show that LSEC contribute to uptake and lysosomal degradation of enterobacterial viruses, such as bacteriophage, acting as a primary anti-viral defense mechanism (Oie et al., 2020). This has implications not only for innate immune responses but also may contribute to the low efficiency of phage therapy, since bacteriophages used for gene delivery appear to be rapidly cleared from the circulation. Furthermore, the morphology of LSEC facilitate immune surveillance against hepatotropic viral infections. Specifically, $CD8^+$ T cells have been shown to extend protrusions through LSEC fenestrae, probing for viral antigens presented by infected hepatocytes (Warren et al., 2006; Guidotti et al., 2015).

Antigen uptake via LSEC scavenger receptors, followed by antigen presentation, is a key step in promoting T cell tolerance under physiological conditions (**Figure 2**). Mannose receptor-mediated uptake, processing and presentation of antigen via major histocompatibility complex (MHC) class I on LSEC facilitates antigen cross-presentation to $CD8^+$ T cells (Limmer et al., 2000; Burgdorf et al., 2006, 2007), inducing tolerance via upregulation of co-inhibitory molecule programmed cell death ligand 1 (PD-L1) (Diehl et al., 2008). This has been demonstrated for both oral (Limmer et al., 2005) and tumour antigens (Berg et al., 2006; Höchst et al., 2012). LSEC can also present antigen to $CD4^+$ T cells via MHC class II, which is constitutively expressed at low levels, but upregulated in response to inflammatory stimuli (Lohse et al., 1996; Knolle et al., 1998). However, the low expression of co-stimulatory molecules in LSEC, particularly in the presence of IL-10, means they are poor stimulators of naïve $CD4^+$ T cell activation (Katz et al., 2004), instead inducing development of regulatory T cells ($T_{reg}$) which suppress immune responses (Carambia et al., 2014). Furthermore, LSEC can impair the ability of DCs to induce naïve T cell proliferation *in vitro*, although the mechanism remains unknown (Bertolino, 2008; Schildberg et al., 2008). LSEC also induce tolerance of recent autoreactive $CD4^+$ thymic emigrants, who acquire IL-10-producing capacity and undergo higher rates of apoptosis via enhanced FasL and Bim expression (Xu et al., 2016). Together, these findings define a key role for LSEC in maintaining peripheral tolerance (**Figure 2**).

Alternatively, LSEC can also present antigen to elicit immunogenic T cell responses (**Figure 2**). For example, LSEC TLR1/2 stimulation with palmitoyl-3-cysteine-serine-lysine-4 (P3C) induced activation of virus-specific $CD8^+$ T cells via low level IL-12 secretion in the absence of PD-L1 expression (Liu et al., 2013). Furthermore, stimulation of NOD1, NOD2 and RIPK2 with diaminopimelic acid (DAP) promotes LSEC maturation and HBV-specific T cell activation (Huang et al., 2018). This effect was mediated by NFκB and mitogen-activated protein kinase (MAPK) activation, and subsequent expression of pro-inflammatory chemokines and cytokines, which ultimately primed HBV-stimulated $CD8^+$ T cells to increase their interferon γ (IFNγ) and IL-2 production.

Equally, high antigen concentrations are sufficient to shift a tolerogenic response to an immunogenic one via excess T cell receptor (TCR) signalling (Schurich et al., 2010). Cross-talk between helper $CD4^+$ and $CD8^+$ T cells is mediated by LSEC, involving simultaneous T cell activation, cross-priming, IL-2 release, TCR stimulation and IL-6 signalling, which ultimately enhances LSEC-primed $CD8^+$ T cell effector ($T_{eff}$) functions (Böttcher et al., 2014; Wittlich et al., 2017). These findings provide evidence that LSEC can switch their homeostatic tolerogenic phenotype to an immunogenic one, promoting T cell immunity in response to microbial antigens. Understanding how LSEC mediate liver-specific tolerance and immunity will have important implications when attempting to overcome T cell tolerance, such as during chronic viral infection or liver cancer.

## Leukocyte Recruitment

LSEC also contribute to another important aspect of immunity, namely the trafficking of leukocytes through recruitment from the peripheral circulation into the liver (**Figure 3**). Leukocyte recruitment follows a multi-step adhesion cascade involving several receptor-ligand interactions, which enable capture of circulating immune cells by activated endothelium, and subsequent transmigration to tissue injury or infection sites (Ley et al., 2007). This process is stimulated by recognition of pattern- and danger-associated molecular patterns (PAMPs/DAMPs) by liver immune sentinels, such as KCs, resulting in their activation and subsequent release of cytokines and chemokines. Generally, initial tethering is mediated by selectins and subsequent rolling by integrin activation, which are dictated by the "catch-bond" phenomenon, whereby receptor-ligand interactions are strengthened under conditions of increased shear stress (Marshall et al., 2003; Yago et al., 2007). This brings about cell arrest, followed by intravascular crawling and transmigration through the endothelium into the tissue sites. Typical adhesion molecules involved in leukocyte recruitment include ICAM-1 and vascular cell adhesion molecule 1 (VCAM-1), which form endothelial adhesive platforms by establishing microdomains in association with tetraspanins (Poisson et al., 2017). For instance, CD151 is a tetraspanin which was shown to associate with LSEC VCAM-1 and mediate lymphocyte adhesion under physiological flow conditions *in vitro* (Wadkin et al., 2017).

In contrast to conventional vascular beds, the low shear flow conditions within the hepatic sinusoids leads to lack of selectin-dependent initial tethering and rolling, paving the way for atypical adhesion molecules (i.e., scavenger receptors) to play a more predominant role (Shetty et al., 2008). The involvement of atypical adhesion molecules in leukocyte recruitment has been previously reviewed (Patten and Shetty, 2018). Scavenger receptors are involved in recruitment of distinct leukocyte subsets, and as such, may prove to be novel liver-specific therapeutic targets (**Figure 3**) (Patten et al., 2019). One example is stabilin-1, which is highly expressed on LSEC in response to hepatocyte growth factor (HGF) and, with the support of adhesion molecules ICAM-1 and vascular adhesion protein 1 (VAP-1), permits the specific transmigration of $T_{reg}$ across the sinusoidal endothelium (Shetty et al., 2011). In addition, scavenger receptor class F, member 1 (SCARF1) mediates selective $CD4^+$ T cell adhesion (Patten et al., 2017a), alongside its scavenging functions in the clearance of LDLs and apoptotic bodies (Patten, 2018).

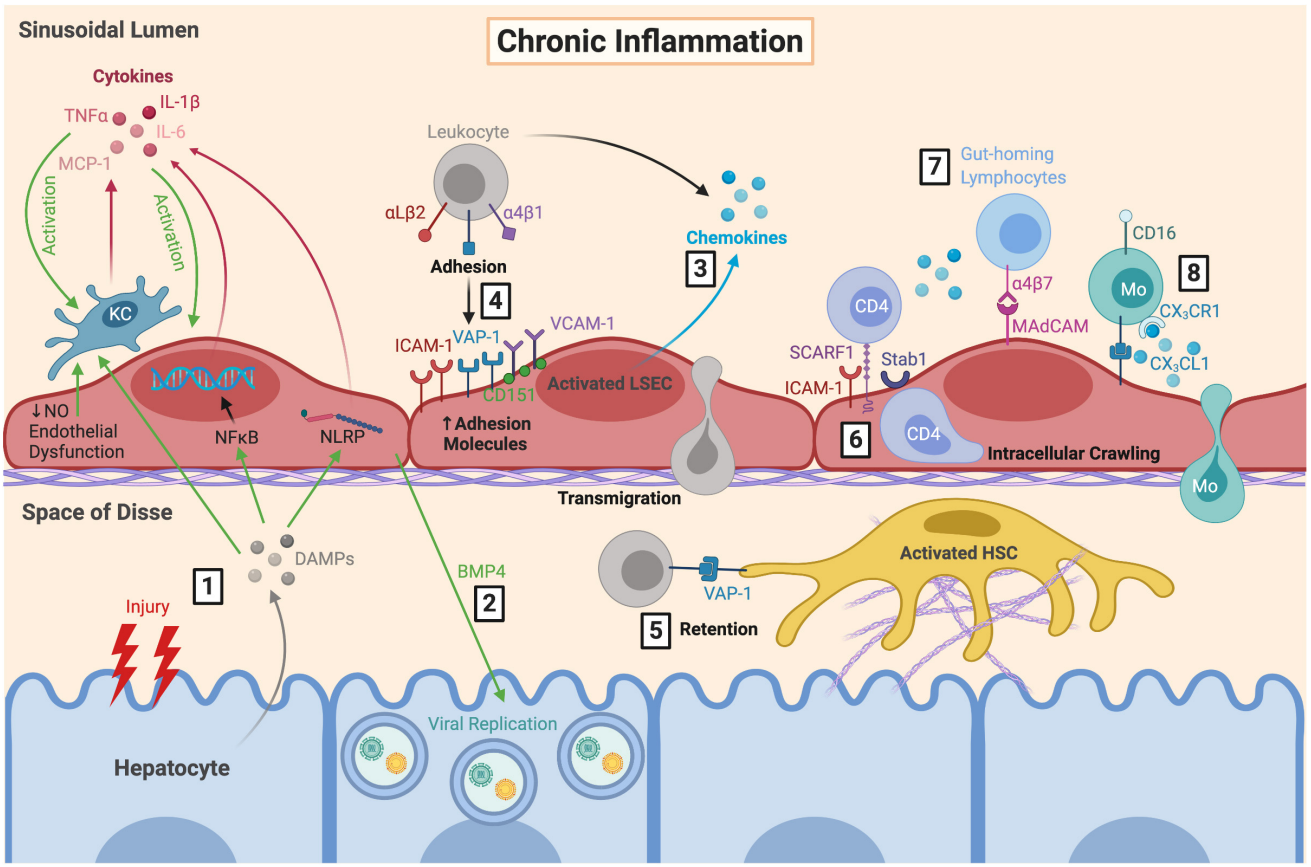


**FIGURE 3 |** LSEC orchestrate the immune microenvironment during chronic inflammation. **(1)** During chronic inflammation, repeated hepatocyte injury results in release of DAMPs which are sensed by KC, resulting in their activation and subsequent production of pro-inflammatory cytokines. These DAMPs also trigger cytokine release from LSEC via NFκB and inflammasome signalling, further perpetuating LSEC and KC activation. This is exacerbated by endothelial dysfunction. **(2)** Production of BMP4 by LSEC can also promote viral replication which can worsen hepatocyte damage during chronic viral infection. Activated LSEC **(3)** secrete chemokines and **(4)** upregulate their expression of adhesion molecules, which facilitates leukocyte recruitment, adhesion and transmigration. **(5)** Leukocytes can be retained within the space of Disse due to VAP-1 expression by HSC. **(6)** Following SCARF1-mediated adhesion, $CD4^+$ T cells have been shown to perform lateral intracellular crawling between LSEC, which is mediated by ICAM-1 and Stab1. LSEC are also important for recruiting distinct pro-inflammatory leukocyte subsets during diseases states, including **(7)** gut-homing lymphocytes via α4β7-MAdCAM interactions, and **(8)** $CD16^+$ Mo via secretion of $CX_3CL1$. LSEC, liver sinusoidal endothelial cells; DAMPs, danger-associated molecular patterns; KC, Kupffer cell; TNFα, tumour necrosis factor α; MCP-1, monocyte chemoattractant protein 1; IL-6, interleukin-6; NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; NLRP, nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain; NO, nitric oxide; BMP4, bone morphogenic protein 4; ICAM-1, intercellular adhesion molecule-1; VAP-1, vascular adhesion protein 1; VCAM-1, vascular cell adhesion molecule 1; SCARF1, scavenger receptor class F member 1; Stab1, stabilin-1; MAdCAM, mucosal addressin cell adhesion molecule 1; Mo, monocyte; $CX_3CL1$, fractalkine; HSC, hepatic stellate cell.

There is also an integral role for chemokines in leukocyte recruitment (**Figures 2**, **3**) (Oo et al., 2010). Chemokines contribute to firm adhesion of leukocytes by binding to G-protein coupled receptors and inducing conformational changes in integrins to facilitate high affinity binding. It is also thought that chemokines are involved in lymphocyte compartmentalisation in liver diseases, with CXCR3 ligands promoting parenchymal recruitment (Curbishley et al., 2005), and CCR5 ligands contributing to recruitment to the portal tracts (Shields et al., 1999; Ajuebor et al., 2004). CXCL9-11 are important for T cell recruitment and transmigration following endothelial activation with IFNγ and TNFα (Curbishley et al., 2005). These chemokine ligands are produced by neighbouring cells and can be transcytosed by LSEC and presented on their cell surface (Middleton et al., 2002; Schrage et al., 2008; Neumann et al., 2015). They can also be circulated within the sinusoids and captured by proteoglycans within the endothelial cell glycocalyx (Curbishley et al., 2005). Fractalkine ($CX_3CL1$) interacts with $CX_3CR1$ on $CD16^+$ monocytes to facilitate adhesion and transmigration in an integrin- and VAP-1-dependent manner (Aspinall et al., 2010). LSEC also express CXCL16 (Geissmann et al., 2005), which interacts with $CXCR6^+$ $T_{eff}$ cells to mediate recruitment (Heydtmann et al., 2005; Sato et al., 2005), as well as natural killer (NK) (Hudspeth et al., 2016; Stegmann et al., 2016) and NKT cells (Geissmann et al., 2005) to promote migration during immune surveillance. Thus, chemokines play an important role in recruiting distinct leukocyte subsets across

LSEC and maintaining the immune microenvironment within the liver.

Hepatocyte paracrine factors can also enhance expression of LSEC adhesion molecules including ICAM-1, VCAM-1 and VAP-1, indirectly regulating immune cell recruitment (Edwards et al., 2005). LSEC adhesion molecules are relevant in tumourigenesis and metastatic spread and their regulation by transformed hepatocytes or metastatic deposits is of interest. For instance, tetraspanin CD151 is upregulated on LSEC by hepatoma-derived factors and collaborates with VCAM-1 to facilitate recruitment (Wadkin et al., 2017). CD151 has been shown to form endothelial adhesive platforms with VCAM-1 and ICAM-1 in human umbilical vein endothelial cells (HUVEC), permitting lymphocyte adhesion and transmigration, although these structures are yet to be identified in LSEC (Barreiro et al., 2005; Barreiro et al., 2008). Following transmigration, there is also evidence to suggest hepatocytes can modulate T cell populations by engulfment and subsequent lysosomal degradation of autoreactive $CD8^+$ T cells (Benseler et al., 2011) and $T_{reg}$ (Davies et al., 2019). These cell-cell interactions have been recently reviewed (Davies et al., 2020).

Although transmigration typically occurs via the paracellular route between endothelial cell junctions, in the liver, lymphocytes frequently extravasate through the endothelial cell body via the transcellular route (Shetty et al., 2011; Patten et al., 2017b). There have also been reports of lymphocyte intracellular crawling within LSEC, shown by live confocal imaging, which was mediated by IFNγ along with ICAM-1 and stabilin-1 expression (Patten et al., 2017b). This demonstrates that LSEC are not just a simple barrier but play an active role in regulating the liver microenvironment. Indeed, the immune microenvironment and leukocyte subsets within it determine the fate of liver injury – resolution, or persistence and chronic hepatitis. Furthermore, excessive immunosuppressive leukocyte subsets promote pathogen escape and tumourigenesis.

## LSEC PATHOPHYSIOLOGY

An expanding body of evidence strongly implicates LSEC in the development of CLD, and thus liver cancer (**Figure 4**), due to the nature of liver disease progression from fibrosis to cirrhosis and HCC development (**Figure 5**). General disease pathways involve chronic liver injury and subsequent endothelial and epithelial damage and dysfunction, which leads to HSC activation, excess ECM deposition, and fibrogenesis. This perpetuates liver damage and can lead to cirrhosis if unresolved (Patten et al., 2019). LSEC contribute to pathogenesis in several ways, by fostering conditions which allow persistence of chronic viral infections and driving processes which initiate and exacerbate fibrosis. These include LSEC capillarisation, characterised by loss of fenestrated morphology and acquisition of vascular phenotype, angiogenesis, and endothelial-to-mesenchymal transition (EndMT). LSEC also release endothelial-derived growth factors, known as angiocrine factors, which determine the balance between regeneration and fibrosis as well as orchestrating tumourigenesis.

### Chronic Viral Infection

As discussed above, LSEC play an important role in viral clearance (**Figure 2**), by direct sensing of viral RNA which can lead to release of type I and III interferon-rich exosomes and inhibition of viral replication (Pohlmann et al., 2003; Broering et al., 2008; Giugliano et al., 2015). LSEC also recruit and position $T_{eff}$ through expression of ICAM-1, VCAM-1 and VAP-1, and presentation of CXCR3 ligands (Curbishley et al., 2005). They also aid retention of $CXCR6^+$ T cells via CXCL16 expression (Heydtmann et al., 2005; Sato et al., 2005).

Conversely, LSEC can also promote hepatotropism of HBV and HCV by permitting them access to the parenchyma, through fenestrations and by transcytosis (Breiner et al., 2001; Protzer et al., 2012). Hence, tolerogenic responses may help viral evasion from the immune system, and LSEC may act as a reservoir for endogenous re-infection. Furthermore, paracrine signals released from LSEC can facilitate HCV replication. One example is bone morphogenic protein 4 (BMP4), whose expression is low in normal liver since it is negatively regulated by VEGFR2 activation, but is upregulated in CLD due to reduced VEGFR2/p38 MAPK signalling (Rowe et al., 2014). LSEC therefore have a pleiotropic role in viral infection, persistence and clearance (**Figure 2**).

### Sinusoidal Capillarisation and Endothelial Dysfunction

Maintenance of the LSEC phenotype is crucial for them to carry out their physiological function and maintain homeostasis (**Figure 1**), yet a common response to chronic injury is the development of capillarisation. Sinusoidal capillarisation is the process by which LSEC lose their fenestrated morphology and adopt a more "capillary-like" phenotype. Capillarisation is associated with basement membrane synthesis, loss of GATA4-dependent signals and upregulation of CD31 and VCAM1 (Xu et al., 2003; Shetty et al., 2018). Following production of substantial basement membrane, the phenotypic alterations in LSEC become virtually irreversible. Capillarisation is analogous with endothelial dysfunction, in which LSEC can no longer maintain HSC quiescence in response to shear stress signals (Deleve et al., 2008; Xie et al., 2012), and together these processes precede fibrosis (Horn et al., 1987; Fraser et al., 1991; Pasarín et al., 2012; Baiocchini et al., 2019). A recent study, however, showed that LSEC dysfunction and loss of fenestrations following chronic metabolic stress do not always go hand in hand (Kus et al., 2019). Specifically, it was shown that mice subject to high-fat diet developed non-alcoholic fatty liver disease (NAFLD)-like disease characterised by steatosis, weight gain, insulin resistance and a pro-inflammatory LSEC phenotype, yet LSEC bioenergetics and fenestrae were functionally preserved. This demonstrates the discernible ability of LSEC to adapt to metabolic stress and pro-inflammatory burden associated with NAFLD. Nevertheless, there is compelling evidence to suggest that capillarisation and endothelial dysfunction not only precede fibrosis, but also promote it (**Figure 5**).

It is well-documented that fenestrations are altered in pathophysiological conditions (Horn et al., 1987; Clark

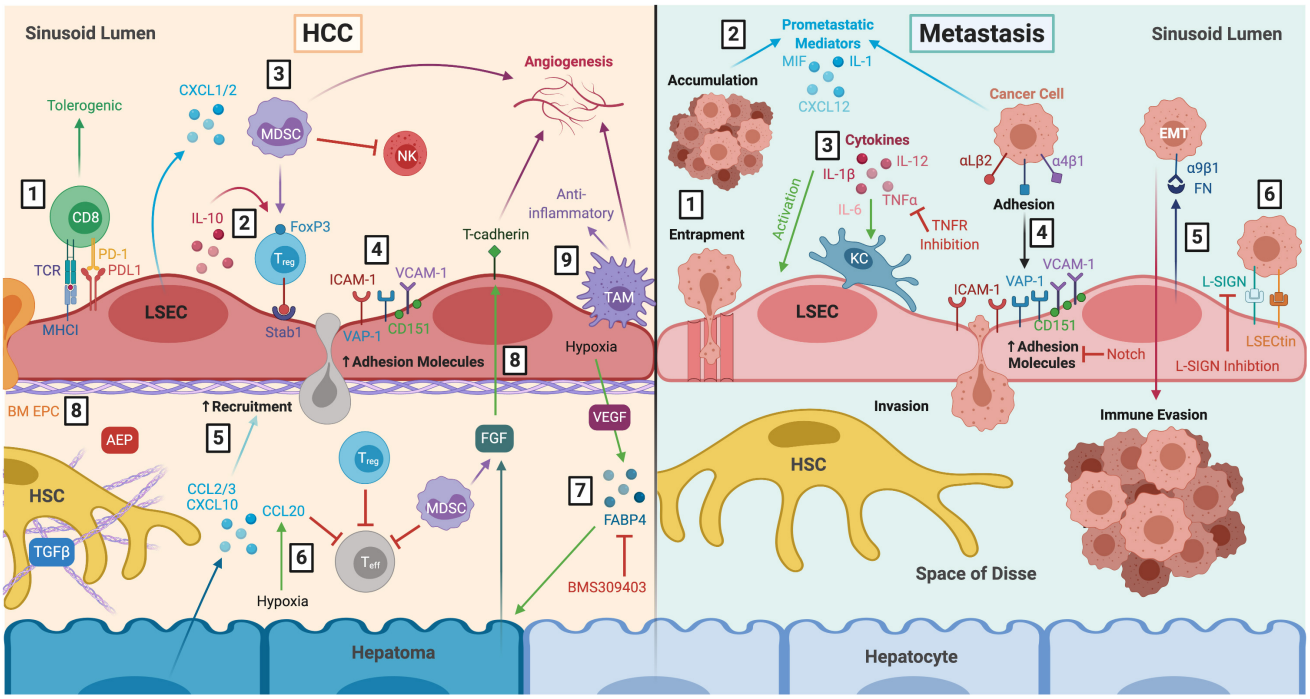


**FIGURE 4 |** LSEC actively contribute to the tumour microenvironment during HCC and liver metastasis. *Left:* LSEC promote an immunosuppressive microenvironment and thus, HCC development and progression. **(1)** LSEC presentation of tumour antigens to $CD8^+$ T cells via MHCI induces tolerogenic responses, in the presence of PDL1, which is upregulated in HCC tumours. **(2)** Production of IL-10 by LSEC induces $T_{reg}$, which are recruited via Stab1, undergoing transmigration and inhibiting $T_{eff}$ responses. **(3)** $T_{reg}$ are also induced by MDSCs, which accumulate in HCC due to LSEC production of CXCL1 and CXCL2. MDSCs elicit pro-tumourigenic effects including inhibition of T cell activation, NK cell inhibition, and stimulation of angiogenesis. **(4)** Transdifferentiated LSEC lose expression of LSEC markers and upregulate expression of adhesion molecules VCAM-1, CD151, VAP-1 and ICAM-1, which facilitates leukocyte recruitment. **(5)** Transformed malignant hepatocytes enhance CCL2, CCL3, and CXCL10 secretion, further promoting leukocyte recruitment. **(6)** Hypoxia-induced production of CCL20 by hepatomas inhibits T cell proliferation. **(7)** LSEC production of adipokines, such as FABP4, in response to hypoxia and VEGF exert pro-oncogenic effects by inducing hepatocyte proliferation. These effects can be attenuated with FABP4-specific inhibitor BMS309403. **(8)** LSEC also foster conditions which promote pro-tumourigenic angiogenesis, including recruitment of pro-angiogenic BM EPC, AEP production and expression of T-cadherin in response to hepatoma- and MDSC-derived FGF. **(9)** Anti-inflammatory TAM also promote immunosuppression and angiogenesis. *Right:* LSEC orchestrate formation of a pre-metastatic niche which promotes development of secondary liver tumours. **(1)** Blood-borne cancer cells can become entrapped within LSEC fenestrae and accumulate in the sinusoidal lumen. **(2)** Tumour cells promote LSEC secretion of pro-metastatic mediators such as MIF, IL-1 and CXCL12, as well as **(3)** activation of KC which produce pro-inflammatory cytokines that in turn activate LSEC. TNFR inhibition has been shown to prevent liver metastasis in mice. **(4)** Activated LSEC upregulate expression of adhesion molecules which promotes binding and invasion of cancer cells to the space of Disse, where they are generally protected from KC and NK cells within the sinusoids. Wnt-independent Notch activation has been shown to inhibit tumour cell adhesion. **(5)** LSEC secrete FN which interact with α9β1 integrin on cancer cells, initiating EMT and promoting metastatic spread. **(6)** LSEC also express L-SIGN and LSECtin, which are upregulated in liver metastasis and mediate adhesion and migration of cancer cells. L-SIGN blockade reduces colon cancer metastasis in murine models. LSEC, liver sinusoidal endothelial cells; HCC, hepatocellular carcinoma; MHCI, major histocompatibility complex I; PD-1, programmed cell death protein 1; PDL1, programmed death ligand 1; IL-10, interleukin 10; $T_{reg}$, regulatory T cell; Stab1, stabilin-1; $T_{eff}$, effector T cell; MDSC, myeloid-derived suppressor cell; CXCL1, C-X-C chemokine ligand type 1; NK, natural killer cell; VCAM-1, vascular cell adhesion molecule 1; VAP-1, vascular adhesion protein 1; ICAM-1, intercellular adhesion molecule 1; CCL2, C-C chemokine ligand type 2; FABP4, fatty acid binding protein 4; VEGF, vascular endothelial growth factor; BM EPC, bone marrow erythroid progenitor cells; AEP, asparaginyl endopeptidase; FGF, fibroblast growth factor; TAM, tumour-associated macrophage; HSC, hepatic stellate cell; TGFβ, transforming growth factor β; MIF, macrophage migration inhibitory factor; KC, Kupffer cell; TNFα, tumour necrosis factor α; TNFR, tumour necrosis factor receptor; FN, fibronectin; EMT, epithelial-to-mesenchymal transition; L-SIGN, lymph node-specific ICAM-3 grabbing non-integrin; LSECtin, lymph node sinusoidal endothelial cell C-type lectin.

et al., 1988; Fraser et al., 1991; Xu et al., 2003; Baiocchini et al., 2019). Fenestrations also decrease with age (Ito et al., 2007), a process dependent on p53 and $p19^{ARF}$- dependent signalling (Koudelkova et al., 2015) which is associated with pseudocapillarisation, sinusoidal dysfunction, loss of vasodilatory capacity, and increased hepatic vascular resistance (Dg et al., 2007; Jamieson et al., 2007; Maeso-Diaz et al., 2018). As eluded to above, fenestrations are dynamic structures which are regulated by several factors and pathways. Studies have shown that cross-talk between LSEC and other hepatic cells can result in loss of fenestrations, for example, LSEC-KC interactions were shown to elicit fenestration loss and upregulation of CD31 (Ford et al., 2015). Additionally, a recent study implicated BMP9, a paracrine factor produced by HSC, as a key regulator of LSEC fenestrations (Desroches-Castan et al., 2019). It was shown that BMP9 maintains vascular quiescence via interactions with its receptor ALK1, and that BMP9 genetic deletion drives LSEC capillarisation and development of perisinusoidal fibrosis in

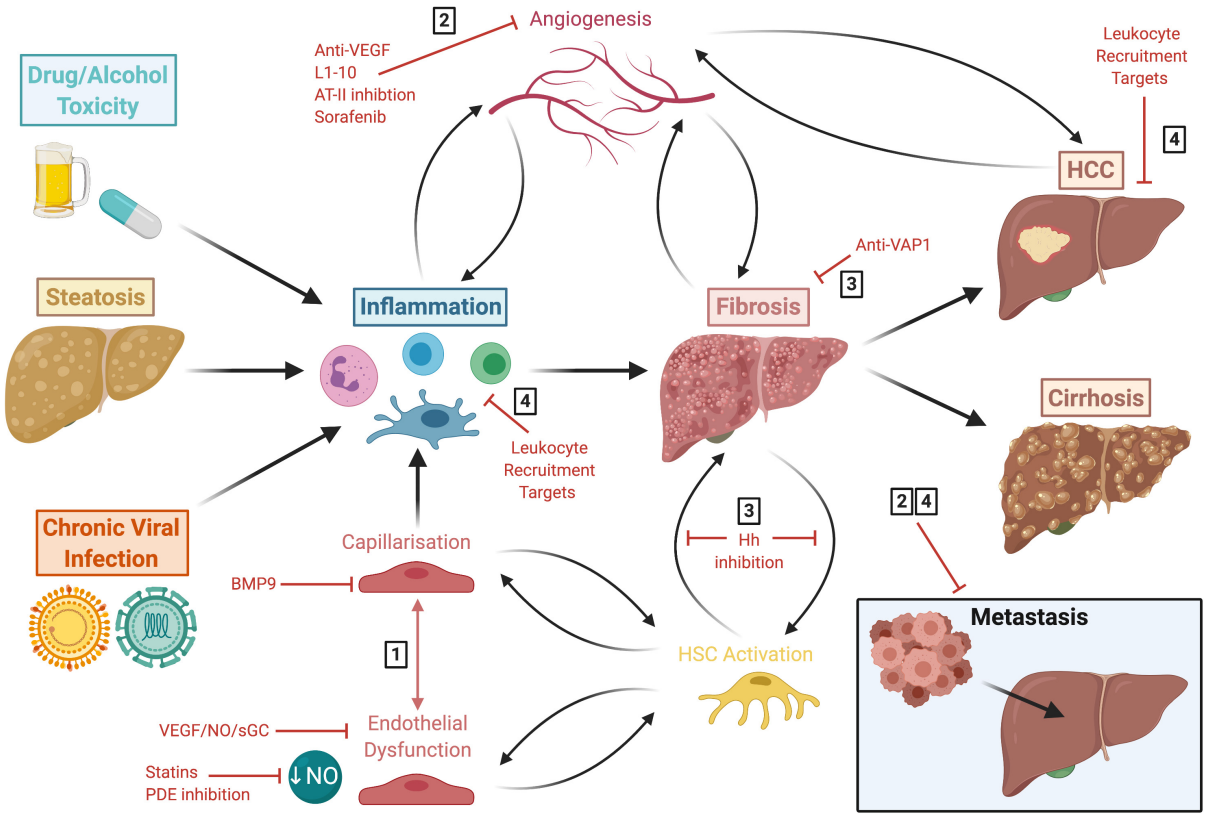


**FIGURE 5 |** Liver disease follows a common pathway of progression which results in fibrogenesis that is both preceded and driven by LSEC capillarisation and dysfunction. This figure summarises the common disease pathways discussed in this review, highlighting various approaches for potential therapeutic intervention. These include: **(1)** molecules which maintain LSEC homeostasis, such as BMP9, statins and phosphodiesterase inhibitors; **(2)** anti-angiogenics which are also anti-inflammatory and anti-fibrotic, such as L1-10, AT-II inhibition and sorafenib; **(3)** anti-fibrotics, including anti-VAP1 and Hh inhibition; **(4)** and targets involved in leukocyte recruitment. LSEC, liver sinusoidal endothelial cells; HCC, hepatocellular carcinoma; BMP9, bone morphogenic protein 9; VEGF, vascular endothelial growth factor; NO, nitric oxide; sGC, soluble guanylate cyclase; PDE, phosphodiesterase; AT-II, angiotensin II; VAP-1, vascular adhesion protein 1; Hh, hedgehog; HSC, hepatic stellate cell.

mice. Moreover, addition of exogenous BMP9 to LSEC primary cultures prevented fenestration loss and preserved GATA4 and PLVAP expression.

Behaviour of endothelial cells is regulated largely by mechanical cues of shear stress which is influenced by blood flow through the lumen of the sinusoids (**Figure 1**). Despite low flow rate, shear stress is generated due to the narrow diameter of the sinusoids. Increasing levels of shear stress result in NO synthesis by LSEC which in turn act to limit vascular resistance by causing vasodilation. Furthermore, the NO signalling pathway maintains the LSEC differentiated phenotype in an autocrine fashion, which is thought to be mediated by the VEGF signalling pathway (DeLeve et al., 2004). Dysfunctional LSEC have impaired eNOS activity, meaning vasodilation switches to vasoconstriction, thus increasing intrahepatic vascular resistance (Rockey and Chung, 1998; Francque et al., 2012). As a compensatory mechanism, LSEC in cirrhotic livers overexpress KLF2 to manage vascular dysfunction, although eventually this is insufficient in preventing portal hypertension and exacerbation of cirrhosis (Gracia-Sancho et al., 2011; Marrone et al., 2013, 2015). Restoration of the NO-dependent pathway via simvastatin (Marrone et al., 2013, 2015; Wang et al., 2013) or sildenafil (Tateya et al., 2011) treatment was shown to improve liver inflammation in rodent steatosis models. Similarly, LSEC differentiation can be re-established by treatment with soluble guanylate cyclase (sGC) activator, BAY-60-2770, which leads to HSC quiescence and attenuation of cirrhosis in rats (Xie et al., 2012).

These pertubations in mechano-sensing by LSEC drive fibrotic processes (Ford et al., 2015; Soydemir et al., 2019) and changes in hepatic blood pressure and liver stiffness occur soon after hepatic injury (Georges et al., 2007). Biophysical characteristics of the ECM and matrix stiffness are key mechanisms in mediating HSC activation contributing to fibrogenesis (Sakata et al., 2004; Olsen et al., 2011). In addition to effects on HSC, mechanical stiffness also impacts hepatocyte phenotype, which is important for regulating cellular responses to tissue injury (Natarajan et al., 2017). Furthermore, mechanical cues are also thought to have indirect effects on LSEC, including cytoskeletal remodelling, loss of fenestrations and formation of stress fibers (Juin et al., 2013; Ford et al., 2015). Hilscher et al. (2019) suggested that activation of PIEZO channels, triggered by integrins and myosin filaments, may be an underlying factor allowing LSEC to respond to

changes in shear stress and pressure. Briefly, authors showed that expression of Notch-dependent transcription factors HES1 and HEY1 resulted in neutrophil recruitment via CXCL1 secretion, a mechanism thought to drive microthrombus formation and portal hypertension in mice. Additionally, activated HSC further increase tissue stiffness by depositing more ECM, further driving mechano-activation (Soydemir et al., 2019). Drugs that intervene with this mechano-sensitive positive feedback cycle could show therapeutic promise for the treatment of fibrosis.

Notch signalling has previously been shown to exacerbate LSEC capillarisation via downregulation of the eNOS/sGC pathway (Duan et al., 2018). Notch ligand DLL4 (delta-like ligand 4), which is upregulated in LSEC from cirrhotic patients and carbon tetrachloride ($CCl_4$)-treated mice, and has been shown to drive loss of fenestrations and deposition of basement membrane (Chen et al., 2019). The overexpression of DLL4 during liver fibrosis was linked to upregulation of ET-1 and enhanced HSC sinusoidal coverage, which was thought to be initiated by hypoxic conditions associated with fibrogenesis. This validates the Notch pathway and its ligands as potential fibrotic targets, since DLL4 knockdown ameliorated LSEC de-differentiation and provided protection against $CCl_4$-induced fibrosis. Contrastingly, Dill et al. (2012) have demonstrated vascular remodelling as a result of disrupted Notch1 signalling, and that maintenance of this signalling pathway conserves the LSEC highly differentiated phenotype. More research is required to fully elucidate the role of Notch signalling in maintenance or alteration of the LSEC phenotype.

An additional pathway that drives LSEC dysfunction is the hedgehog signalling pathway (Xie et al., 2013). Production of hedgehog molecules and other mediators (e.g., transforming growth factor β (TGFβ), laminin and fibronectin) by LSEC activate HSC, which in turn produce hedgehog-containing microparticles that interact with LSEC to further enhance hedgehog signalling (Witek et al., 2009; Xie et al., 2013). Thus, inhibition of hedgehog signalling prevents LSEC capillarisation and restores the differentiated LSEC phenotype (Xie et al., 2013; Zhao et al., 2017). Collectively, these findings exemplify the intimate cross-talk between LSEC and HSC, and the vicious cycle of endothelial dysfunction and HSC activation, which perpetuates fibrogenesis (**Figure 5**).

## Angiogenesis

The formation of new blood vessels, known as angiogenesis, is a key feature of CLD and HCC that is often associated with areas of fibrogenesis (Coulon et al., 2011; Paternostro et al., 2010). Whilst angiogenesis is a physiological process that is crucial for maintaining homeostasis, in the context of inflammation and endothelial dysfunction, angiogenesis becomes pathological in that it exacerbates fibrotic processes. It is thought that LSEC capillarisation and dysfunction precedes fibrogenesis, that in turn drives angiogenesis, which ultimately perpetuates inflammation and fibrosis (**Figure 5**) (Kitade et al., 2008, 2009). This is exemplified by the anti-fibrotic action of anti-angiogenic drugs.

Chronic inflammation promotes angiogenesis by several mechanisms, including sustaining hypoxia and inducing transcription of angiogenic hypoxia-inducible factor 1α (HIF1α)-dependent genes and VEGF (Hammoutene and Rautou, 2019). LSEC actively participate in pathological hepatic angiogenesis by releasing pro-angiogenic factors VEGF (Yoshiji et al., 2003), angiopoietins (Taura et al., 2008; Lefere et al., 2019) and adipokines (Kitade et al., 2006) in response to hypoxia, liver injury, inflammation and fibrosis (Zhang et al., 2015). It is known that NRP-1 initiates pro-fibrogenic signalling by promoting HSC activation. A recent study has also implicated NRP-1 in angiogenesis during liver cirrhosis, by upregulation of VEGFR2 expression and activation via PI3K/Akt signalling in LSEC (Wang et al., 2019). Interestingly, NRP-1 and VEGFR2 complex with PLVAP during foetal angiogenesis (Auvinen et al., 2019); whether PLVAP may also drive angiogenesis in adult liver remains to be determined. CD147 has also been shown to promote fibrosis by enhancing hepatic angiogenesis via VEGF-VEGFR2 signalling, which mediated hepatocyte-LSEC cross-talk (Yan et al., 2015). However, when targeting VEGF signalling it should be borne in mind the cell- and context-dependent effects of this approach, since inhibition of VEGFR2 in myeloid cells could both prevent angiogenesis and fibrosis whilst simultaneously hindering LSEC degradation of the ECM and fibrosis resolution (Yang et al., 2014; Kantari-Mimoun et al., 2015).

Angiopoietin-2/Tie2 interactions have been implicated in pathological angiogenesis in non-alcoholic steatohepatitis (NASH), since peptibody L1-10 reduced hepatic angiogenesis and restored normal vascular microarchitecture. In addition, L1-10 treatment downregulated endothelial adhesion molecules VCAM-1, ICAM-1 and monocyte chemoattractant protein 1 (MCP-1), which was also observed in other CLD models including $CCl_4$ treatment and bile duct ligation (Lefere et al., 2019). In the same respect, angiotensin II (AT-II) receptor inhibition with candesartan inhibits liver angiogenesis and fibrosis (Yoshiji et al., 2006; Tamaki et al., 2013), whilst anti-VEGFR2 antibodies normalise liver vasculature and reduce inflammatory gene expression in the liver (Coulon et al., 2013). The natural anti-fibrotic compound, Fuzheng Huayu, has been shown to mitigate $CCl_4$-induced sinusoidal capillarisation, angiogenesis and expression of angiogenic factors CD31, VEGF, VEGFR2, pERK, and HIF-1α, ultimately reducing liver injury and fibrosis in $CCl_4$-treated mice (Liu et al., 2019). Furthermore, targeting of VEGF expression by HSC using compounds such as curcumin (Zhang et al., 2014) and nintedanib (Ozturk Akcora et al., 2017) has also shown to attenuate fibrosis. Inhibition of hedgehog signalling with tetramethylpyrazine reduced angiogenesis and alleviated fibrosis *in vitro* and *in vivo* (Zhao et al., 2017). This was thought to be mediated, at least in part, by restoration of LSEC fenestration and decreased expression of angiogenic markers VEGFA and VEGFR2 as well as endothelial markers CD31 and CD34. These data further support the targeting of angiogenesis to elicit anti-inflammatory and anti-fibrotic effects.

## Angiocrine Factors

Angiocrine factors produced by endothelial cells mediate organ homeostasis, self-renewal and stem cell differentiation, as well as orchestrating tumour growth and metastasis (**Figure 4**) (DeLeve, 2013). LSEC production of angiocrine signals is tightly regulated

and determines the balance between regeneration and fibrosis in response to acute and chronic liver injury. These are mediated by the CXCR7-Id1 and CXCR4 pathways, respectively (Ding et al., 2014). Indeed, poor hepatocyte regeneration correlates with both cellular and functional loss of liver endothelial cells and a decrease in CXCR7-Id1 and HGF expression during acute-on-chronic liver failure (Shubham et al., 2019).

LSEC release HGF and Wnt2 which regulate the functional maintenance and regeneration of hepatocytes. These angiocrine factors are regulated by the VEGF-Id1 axis, since HGF and Wnt2 are not upregulated following partial hepatectomy in Id1 knockout mice (Ding et al., 2010). Further, treatment of LSEC with mitogenic neuropeptide substance P was shown to not only improve endothelial cell viability, proliferation and production of NO/HGF, but also ameliorated TNFα-induced endothelial dysfunction and promoted hepatocyte regeneration (Piao et al., 2019). Interestingly, there is some evidence to suggest that it is not mature LSEC which drive regeneration in the liver, but rather bone marrow-derived progenitor cells of the sinusoidal endothelium. VEGF-SDF1α signalling following liver injury or partial hepatectomy results in recruitment of $CXCR7^{+}$ bone marrow-derived sinusoidal endothelial progenitor cells which mediate liver regeneration (DeLeve et al., 2016).

## Endothelial-to-Mesenchymal Transition

Alongside LSEC phenotypic changes and angiogenesis, there is also evidence to suggest that fibrosis may be driven by endothelial-to-mesenchymal transition (EndMT). The mechanisms by which endothelial cells convert into myofibroblasts is referred to as EndMT, which contributes to ECM deposition and fibrogenesis in liver disease. Healthy LSEC produce modest amounts of collagen type IV and fibronectin under steady state conditions. During liver fibrosis, ECM production increased several-fold but the composition remains relatively consistent (Natarajan et al., 2017). Exposure of LSEC to interstitial collagen fibers and laminin results in defenestration (McGuire et al., 1992; Shakado et al., 1995). Interestingly, culture of LSEC on decellularised liver ECM maintained their fenestrated phenotype for longer periods compared to ECM from other organs (Sellaro et al., 2007).

The production of TGFβ, collagens, fibronectin and laminin by capillarised LSEC may be considered EndMT (Maher and McGuire, 1990; Neubauer et al., 1999), a process characterised by co-expression of CD31 and α-sma (Ribera et al., 2017). EndMT occurs when endothelial cells undergo a series of molecular events and gain a mesenchymal (e.g., myofibroblastic) phenotype, and is a characteristic of many fibrotic diseases (Piera-Velazquez et al., 2016), including cardiovascular and pulmonary disease. However, only a handful of studies have demonstrated EndMT *in vivo* in cirrhotic patients (Dufton et al., 2017; Ribera et al., 2017). These have been validated in $CCl_4$ mouse models, where it has been shown that EndMT is mediated by TGFβ – SMAD3 signalling and can be attenuated by BMP7 and etanercept (TNFα inhibitor) treatment (Dufton et al., 2017; Ribera et al., 2017). Decreased EndMT in response to BMP7 treatment correlated with attenuated fibrosis and improved vascular disorganisation (Ribera et al., 2017).

Li Z. et al. (2019) recently showed that inhibition, or endothelial-specific deletion, of transcriptional modulator MKL1 suppresses TGFβ-induced EndMT and associated fibrosis. The driving effect of MKL1 on EndMT was shown to be mediated by recruitment to the promoter region of TWIST1, activating its transcription in a STAT3-dependent manner. Both STAT3 and TWIST1 inhibition, with C188-9 and harmine, respectively, reversed EndMT and bile duct ligation-induced fibrosis in mice, proposing the STAT3-MKL1-TWIST1 axis as a novel fibrogenic pathway with potential for therapeutic targeting. In contrast, endothelial transcription factor (ETS)-related gene (ERG) protects against EndMT by preferentially driving SMAD1 signalling and repressing SMAD3 activity, whilst ERG genetic ablation drove EndMT and spontaneous liver fibrosis (Dufton et al., 2017). Furthermore, decreased ERG expression correlated with EndMT in end-stage liver disease patients (Dufton et al., 2017), suggesting it could be a valid biomarker for assessing EndMT in liver disease.

Clinical studies and animal models suggest that fibrosis can be reversible (Soyer et al., 1976; Hammel et al., 2001; Arthur, 2002; Dixon et al., 2004; Issa et al., 2004). One therapeutic approach is aimed at degradation of the ECM through targeting of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) (Clutterbuck et al., 2009; Roderfeld, 2018). Chronic hepatitis patients have lower circulating levels of collagenase, MMP-1, and excess TIMP-1 which positively correlates with aminotransferase levels and fibrosis score (Ninomiya et al., 2001; Flisiak et al., 2004; Guido et al., 2006). Increasing the MMP-1/TIMP-1 ratio has shown promise in chronic hepatitis B and C patients (Ninomiya et al., 2001; Flisiak et al., 2004; Guido et al., 2006). Further, studies in rats have shown that transient overexpression of MMP-1 decreases type I collagen, induces hepatocyte proliferation, and attenuates fibrosis (Iimuro et al., 2003), whilst anti-TIMP-1 antibody treatment reduced collagen accumulation and α-sma expression (Parsons et al., 2004). This is interesting as LSEC, along with HSC, possess MRs which can endocytose denatured collagen α chains from the circulation and space of Disse (Malovic et al., 2007; Madsen et al., 2012). However, MMPs are also implicated in fibrogenesis; their pleiotropic roles in fibrosis have been systematically reviewed (Hemmann et al., 2007). For instance, in mice, MMP-9 has been implicated in activation of latent TGFβ, a process which can drive HSC activation and subsequent collagen deposition, thereby promoting fibrosis (Yu and Stamenkovic, 2000). Nevertheless, it seems that LSEC which have undergone EndMT contribute to fibrogenesis, whilst LSEC which maintain their differentiated phenotype contribute to fibrinolysis and resolution.

## Autophagy

The process of EndMT and TGFβ signalling have been linked to autophagy which is the process of regulated degradation and recycling of intracellular components. Upregulation of TGFβ signalling and EndMT can be induced by loss of autophagy genes, *ATG7* and *ATG4B*, which has been shown to exacerbate inflammation and fibrosis in murine models of cardiac (Li et al., 2016), pulmonary (Cabrera et al., 2015; Singh et al., 2015), renal

(Nam et al., 2019a,b), and pancreatic (Zhou et al., 2017) fibrosis. Conflictingly, autophagy has also been defined as a driver of fibrotic disease (Hernandez-Gea et al., 2012; Ghavami et al., 2015; Livingston et al., 2016). Ruart et al. (2019) investigated the role of autophagy in regulating endothelial dysfunction during liver injury. The authors showed that LSEC homeostasis is maintained by autophagy, a process which protects against oxidative stress, that is rapidly upregulated following capillarisation *in vitro* and *in vivo*. Selective loss of endothelial autophagy resulted in reduced intrahepatic NO and subsequent cellular dysfunction which perpetuated fibrosis in $CCl_4$-treated mice.

In keeping with these findings, defective autophagy pathways in LSEC have been shown to occur in NASH patients compared with controls and steatotic patients. Autophagy deficiency was linked to liver inflammation, EndMT, apoptosis and perisinusoidal fibrosis in a mouse model of NAFLD, outcomes that were independent of metabolic risk factors such as body weight and plasma cholesterol (Hammoutene et al., 2020). Furthermore, activation of autophagy with hypercholesterolaemia drug ezetimibe, via AMPK activation and nuclear translocation of transcription factor TFEB, ameliorated steatohepatitis by dampening inflammasome signalling in macrophages (Kim et al., 2017). In contrast, autophagy in HSC has been shown to release lipids that promote fibrogenesis (Hernandez-Gea et al., 2012). Thus, the role of autophagy in hepatic fibrosis seems to be cell-specific, with LSEC (Ruart et al., 2019), macrophage (Kim et al., 2017), and hepatocyte (Singh et al., 2009) autophagy providing a protective role, and autophagy in HSC proving detrimental (Hernandez-Gea et al., 2012). This should be considered when designing compounds which target autophagy for fibrotic indications.

## LSEC IN THE TUMOUR MICROENVIRONMENT

Both the structure and function of LSEC render them capable of playing an active role in contributing to the tumour microenvironment (TME) and in the development of primary liver cancer (**Figure 4**). Of note, the liver is a strongly immunosuppressive environment which acts as protection against inflammation derived from gut antigens. However, the same mechanisms that prevent liver inflammation can foster conditions which promote tumour development.

As discussed previously, LSEC are able to cross-present soluble antigen on their MHCI to $CD8^+$ T cells. Due to co-inhibitory signalling, $CD8^+$ T cells initially activated by LSEC regain a quiescent state in which they are unable to exert cytotoxic effects upon circulating tumour cells or antigens (Diehl et al., 2008). Thus, LSEC induce tolerance of $CD8^+$ T cells toward antigens (Limmer et al., 2000). Moreover, Berg et al. (2006) demonstrated that LSEC are able to induce tolerance toward tumour cells, and Höchst et al. (2012) found that LSEC are involved in tolerance toward carcinoembryonic antigen (CEA), which is associated with colorectal carcinoma. Another mechanism by which the liver sinusoids might be pro-tumourigenic is via the TGFβ-dependent induction of $T_{reg}$ (Carambia et al., 2014).

LSEC transdifferentiation and the loss of several LSEC markers are hallmarks of HCC (**Figure 4**). Lymphocyte recruitment is promoted by the expression of ICAM-1, VAP-1 and CD151 (Wadkin et al., 2017), and $T_{reg}$ are specifically recruited by stabilin-1 (Shetty et al., 2011). Hepatocyte malignant transformation enhances the secretion of chemokines (Yoong et al., 1999) (including CXCL10, CCL2, and CCL3) and adhesion molecule expression (Yoong et al., 1998) (ICAM-1 and VAP-1) by LSEC, which in turn promotes leukocyte recruitment. As such, HCC tumour tissue is characterised by $CD8^+$, $CD68^+$, and $FoxP3^+$ immune cell infiltrate, particularly within the invasive margin. The expression of PD-L1 on LSEC correlated with both the incidence of $CD8^+$ T cells and poor survival outcomes (Ihling et al., 2019). This suggests that HCC may evade immune responses via the upregulation of PD-L1 in response to pre-existing cytotoxic T-lymphocyte activity.

The distinction in the TME between primary liver cancer, which usually arises on a background of chronic liver inflammation, and liver metastases, which arise from an otherwise healthy liver, has been recently reviewed (**Figure 4**) (Eggert and Greten, 2017). In HCC, both the tumour itself and the underlying chronic inflammation contribute to the TME. Hypoxia, for example, an early event in the development of liver cirrhosis, promotes an immunosuppressive microenvironment. This is relevant in tumourigenesis, where hypoxia induces tumour cells to secrete CCL20, which subsequently inhibits T cell proliferation (Ye et al., 2016). A recent study also highlighted the key role that LSEC play in balancing hepatic immune cell populations through chemokine presentation (Ma et al., 2018). In this study the investigator showed that LSEC presentation of the chemokine CXCL16 was critical in regulating the anti-tumour response of NKT cells in models of primary and metastatic liver cancer.

One of the earliest mechanisms by which the liver is primed for metastasis is by the accumulation of myeloid cells and myeloid-derived suppressor cells (MDSCs) in the liver (Connolly et al., 2010). MDSCs are immature cells of myeloid origin which have been shown to accumulate in the TME of HCC (Hoechst et al., 2008) and contribute significantly to promoting an immunosuppressive niche (Gabrilovich, 2017). MDSCs exhibit pro-tumoural and pro-metastatic effects via a number of diverse mechanisms, including suppression of T cell activation, inhibition of NK cell activity and induction of $T_{reg}$ (Gabrilovich, 2017; Millrud et al., 2017). MDSCs also promote tumourigenesis by remodelling of the TME and tumour angiogenesis via production of VEGF, basic fibroblast growth factor, Bv8, and MMP9 (Ley et al., 2007; Millrud et al., 2017). Chemokines including CXCL1 and CXCL2 are secreted by LSEC, which are involved in the recruitment of MDSCs to the tumour microenvironment (Brodt, 2016). In HCC, MDSC incidence within tumours directly correlated with disease progression and mortality (Lu et al., 2019).

### Metabolism in HCC

In the last few years there has been major interest in the role of metabolism in regulating immunity. It is now accepted that metabolic pathways shape immune responses rather than

having a mere bystander role. In turn, metabolic factors, such as altered adipokine signalling and lipid metabolism observed in NASH-associated cirrhotic patients, are linked to inflammation-induced cancer. For instance, linoleic acid accumulation mediates selective loss of intrahepatic $CD4^+$ T cells by inducing oxidative stress, which in turn accelerates HCC growth due to impaired anti-tumour surveillance (Ma et al., 2016). The metabolic role of LSEC in lipid metabolism is also likely to contribute to the development of HCC. For example, fatty acid binding protein 4 (FABP4), produced by LSEC in response to hyperglycaemia, VEGF and hypoxia, is increased in NAFLD and HCC patients (Milner et al., 2009; Laouirem et al., 2019). This adipokine exerts pro-oncogenic effects by inducing hepatocyte proliferation, thus enabling HCC to develop in the NAFLD setting (Laouirem et al., 2019). Whilst metformin treatment reduced FABP4 upregulation *in vitro*, specific FABP4 inhibitor BMS309403 reduced tumour growth *in vivo* in murine xenograft models (Laouirem et al., 2019). Adipokines are also known to induce angiogenesis which promotes HCC growth, further highlighting them as a potential target for HCC treatment on a background of NASH. More work is still required to understand how the well-established metabolic role of LSEC impacts the immune and angiogenic environment that supports tumour development within the liver.

## Angiogenesis in HCC

Angiogenesis is a hallmark of all neoplasia, as it facilitates tumourigenesis and metastasis; liver tumours are known to be especially vascularised. During early HCC, there is a switch in tumour blood supply from the portal vein which supplies dysplastic and regenerative nodules as well as 70% of the blood supply to a healthy liver, to the hepatic artery (Semela and Dufour, 2004). There is a growing body of evidence to suggest that LSEC have a role in angiogenesis in the development these hypervascular tumours. HCC tumour progression is associated with phenotypic changes in peri-tumoural LSEC and increased production of angiogenic factors.

There is a strong correlation between changes in LSEC gene expression and angiogenesis in HCC. Endothelial alterations within and proximal to the tumour are associated with HCC. There is a correlation between HCC and a loss of LSEC markers including stabilin-1, stabilin-2, LYVE-1 and CD32b, whilst increasing expression of integrins and ICAM-1 and capacity for angiogenesis, coagulation and fibrinolysis (Wu et al., 2008). Stabilin-2 is an LSEC marker protein; loss of stabilin-2 expression in the endothelial cells of peri-tumoural tissue conferred a significant overall survival advantage (Géraud et al., 2013). It was suggested that this might be via prevention of vascular remodelling and endothelial cell transdifferentiation. Additionally, asparaginyl endopeptidase (AEP), a molecule known to regulate tumour angiogenesis, is absent or low in normal tissues. However, it has found to be significantly upregulated in solid tumours and surrounding ECM, promoting LSEC angiogenesis *in vitro* (Li N. et al., 2019). Therefore, AEP may represent a novel target for progression of CLD and HCC.

The highly angiogenic nature of HCC is associated with increased classical growth factors such as VEGF and PDGF, whilst classical adhesion molecules such as ICAM-1 and VCAM-1 are preferentially expressed on tumour tissue. It has been shown that bone marrow-derived erythroid progenitor cells (BM-EPCs) play a prominent role in HCC angiogenesis (Zhu et al., 2012). LSEC expression of distinct adhesion molecules and growth factors could drive BM-EPCs recruitment, especially since BM-EPC homing to tumour tissue is thought to be via cellular adhesion molecules ICAM-1, VCAM-1, and VEGF.

Another growth factor, FGF2, secreted by HCC and expressed preferentially in tumour tissue compared to CLD, induces T-cadherin on LSEC. T-cadherin is selectively over-expressed in intra-tumoural capillary endothelial cells in many HCC specimens (Adachi et al., 2006). *In vitro*, T-cadherin was found to influence the invasive potential of HCC (Riou et al., 2006), via binding of adiponectin and activation of NFκB, thus preventing tumour cell apoptosis. This suggests T-cadherin might be a mediator of angiogenesis in HCC. Another suggested mediator of angiogenesis is the leukocyte cell-derived chemotaxin 2 (LECT2)-Tie1 signalling pathway, which possesses pleiotropic effects in HCC. On one hand, loss of LECT2 promotes inflammatory monocyte recruitment, suggesting its activity supports an immunosuppressive TME, whilst on the other it acts as a tumour suppressor by inhibiting vascular invasion (Chen et al., 2014). In the context of CLD, LECT2-Tie1 signalling is an important driver of fibrosis by promoting LSEC capillarisation and inhibiting portal angiogenesis (Xu et al., 2019). Consistent with these findings, serum levels of LECT2 are significantly elevated in cirrhotic and HCC patients (Slowik et al., 2019) and correlate with fibrosis stage (Xu et al., 2019), highlighting its usefulness as a potential biomarker or therapeutic target for these indications (Su and Iwakiri, 2020).

Targeting angiogenesis has shown promise in the management of cancer, and current medical therapies focus on this aspect in the setting of HCC. For many years, sorafenib represented the only medical treatment for HCC and increased median survival in advanced HCC by 3 months (SHARP trial) (Llovet et al., 2008). Sorafenib is a multi-kinase inhibitor which acts on VEGF, PDGF and Raf. After a decade as the only approved oral agent for HCC, a second multi-kinase inhibitor, lenvatinib, has now shown to be non-inferior to sorafenib (Kudo et al., 2018). There have been many pre-clinical studies to assess how combinations of anti-angiogenics and other agents may boost therapeutic efficacy. For example, sorafenib inhibits the formation of pre-neoplastic lesions in rat NAFLD models, and combination with AT-II receptor antagonist losartan also had this effect (Yoshiji et al., 2014). AT-II inhibition reduces HIF-1α activity and expression of VEGF which prevents angiogenesis and HCC development in rats (Yoshiji et al., 2006; Tamaki et al., 2013). Inhibition of angiopoietin-2 with L1-10 peptibody reduced angiogenesis, ameliorated steatohepatitis and prevented NASH-associated HCC progression in mice (Lefere et al., 2019). Furthermore, leptin-mediated angiogenesis is known to regulate HCC development and progression

(Kitade et al., 2006), and so targeting this adipokine may represent a potential additional anti-angiogenic approach. A key breakthrough in the rationale of targeting vasculature, such as LSEC, in tumour development has come from studies combining immunotherapy and anti-angiogenics, with major interest in the combination of checkpoint inhibitors and anti-VEGF therapies (Hato et al., 2016).

## LSEC IN LIVER METASTASIS

The liver is also the main site of metastasis from a number of primary tumours, including colon, pancreatic and lung (Budczies et al., 2015; Mielgo and Schmid, 2020). The presence of liver metastases is a poor prognostic marker and therefore, there is an urgent need to understand how liver metastases develop, and subsequently identify potential therapeutic targets.

There are four main stages in the development of liver metastases: (i) the microvascular phase, (ii) the extravascular phase, (iii) the angiogenic phase, and (iv) the growth phase. It has been established that tumours "prime" the sites of metastasis. The role of the TME is well-established in liver metastasis, as is the concept of the pre-metastatic niche, in which the TME creates a prime setting for metastases to seed (**Figure 4**) (Van den Eynden et al., 2013; Brodt, 2016; Mielgo and Schmid, 2020).

LSEC, KC, and NK cells are the first barrier encountered by circulating metastatic cells, forming a natural defense against seeding blood-borne cancer cells. The physical entrapment of tumour cells in the fenestrae of LSEC leads to mechanical stress and deformation. Larger clumps of tumour cells can become trapped in sinusoidal vessels, resulting in localised ischemia-reperfusion, thus triggering the release of NO (Wang et al., 2000) and reactive oxygen species (ROS) from both LSEC and KC (Yanagida et al., 2006). This subsequently results in widespread apoptosis; it has been suggested that up to 95% of circulating tumour cells which encounter LSEC undergo apoptosis (Vekemans et al., 2004).

However, LSEC represent a double-edged sword in liver metastasis. They possess a number of pro-metastatic qualities. Tumour cells promote KC secretion of pro-inflammatory cytokines including TNFα and IL-1. These cytokines stimulate LSEC to upregulate the expression of cellular adhesion molecules including ICAM-1, VCAM-1, endothelial (E)-selectin, and CD31 (Clark et al., 2016). These cellular adhesion molecules can facilitate cancer cell migration to the space of Disse, where there is relative protection from NK and KC (Glinskii et al., 2005). Specifically, cellular adhesion molecules enable tumour cell attachment to LSEC, which in turn promotes tumour cell arrest, extravasation and ultimately, the development of metastasis (Auguste et al., 2007). The proposed mechanisms by which these events occur are detailed below.

### The Role of ICAM-1

Metastatic growth is dependent on tumour-host cell interactions and LSEC are known as key players in this cross-talk. One adhesion molecule shown to be involved in this cross-talk is ICAM-1, which is the main cellular adhesion molecule expressed on LSEC, KC, HSC, and hepatocytes (Zhu and Gong, 2013). ICAM-1 is upregulated by pro-inflammatory cytokines including TNFα, IL-1β, and IFN-γ.

ICAM-1 is a key player in the metastatic process in a number of organs, including lung, liver and blood (Christiansen et al., 1996; Kotteas et al., 2013). It is involved in the first stage of metastasis: adhesion of tumour cells to the endothelial cell wall (in the case of the liver, adhesion of tumour cells to the surface of LSEC). Its expression promotes the activation of pro-metastatic signalling pathways involving IL-6 and IL-8. These pro-inflammatory cytokines increase vascular permeability and further facilitate tumour cell adhesion to the endothelium, thus forming a positive feedback loop promoting tumour seeding and growth. ICAM-1 is also involved in tumour cell extravasation via remodelling of the actin cytoskeleton (Benedicto et al., 2017). *In vivo*, ICAM-1 is implicated in the formation of liver metastases from colorectal cancer (Benedicto et al., 2019), and mediates the infiltration of tumour cells into tumour mass.

### The Role of E-Selectin

The role of sinusoidal E-selectin has been examined by a number of studies. E-selectin mediates the interaction of tumour cells with endothelial cells and is thought to be a critical molecule in tumour adhesion leading to the formation of metastases (Aychek et al., 2008; Tremblay et al., 2008). This correlates clinically; there is increased E-selectin expression found around liver metastases from colorectal primary tumours (Ye et al., 1995). Alexiou et al. (2001) found that elevated levels of E-selectin, ICAM-1 and VCAM-1 correlated with disease outcome in colorectal cancer.

### The Role of Other LSEC-Derived Molecules

It has been shown that LSEC-derived cytokines, including IL-1, macrophage migration inhibitory factor (MIF) and CXCL12 are pro-metastatic (Arteta et al., 2010; Mendt and Cardier, 2017). LSEC secrete fibronectin, which interacts with integrin α9β1 on the surface of colorectal cancer cells, and induces epithelial-to-mesenchymal transition (EMT) via upregulation of Rac signalling pathways and activation of focal adhesion kinase. This mechanism promotes the metastatic capability of colorectal tumour cells (Ou et al., 2014). Kitakata et al. (2002) found that TNFR1 (TNFα receptor)-deficient mice were 50% less likely to develop liver metastases compared to wild-type mice. Wild-type mice injected with tumour cells were found to have significantly increased expression of VCAM-1 and E-selectin on LSEC. Therefore, it is postulated that TNFα plays a key role in the development of liver metastases via its upregulation of VCAM-1 and E-selectin on LSEC.

LSECtin, a C-type lectin, is an adhesion molecule mediating interaction between LSEC and activated T cells. LSECtin is upregulated in liver metastases from colorectal cancer. LSECtin expression correlates with colonic tumour progression and the development of hepatic metastases, which is proposed to be via c-Met upregulation (Zuo et al., 2013). Additionally, lymph

node-specific ICAM-3 grabbing non-integrin (L-SIGN/DC-SIGNR/CLEC4M) and LSECtin have been implicated in adhesion and migration of colon cancer cell metastasis to the liver (Liu et al., 2004; Zuo et al., 2013; Na et al., 2017).

## TARGETING LSEC IN INFLAMMATION AND CANCER THERAPY

As detailed in the above paragraphs, LSEC are central to co-ordination of the inflammation-cancer axis in the liver (**Figure 5**). Since alteration in the LSEC phenotype is one of the earliest events in fibrogenesis, and LSEC are known to both perpetuate inflammation and foster the development of primary and secondary liver tumours, they make an attractive target for CLD and oncotherapy. Indeed, there have been recent advancements in this field, notably in approaches aimed at targeting (i) LSEC dysfunction and capillarisation, (ii) angiogenesis and angiocrine signalling, and (iii) leukocyte and/or tumour cell adhesion and transmigration.

Aforementioned approaches which could maintain LSEC differentiation and function include restoration of their fenestrated phenotype, via BMP9 and GATA4 (Geraud et al., 2017; Desroches-Castan et al., 2019), re-constitution of the VEGF/NO/sGC signalling pathway (Tateya et al., 2011; Xie et al., 2012; Marrone et al., 2013, 2015; Wang et al., 2013), and re-establishment of normal hedgehog signalling with hedgehog inhibitors such as tetramethylpyrazine (Zhao et al., 2017). Furthermore, maintenance of autophagy pathways, which are known to be important for endothelial homeostasis and are defective in CLD patients (Hammoutene et al., 2020), may offer an additional treatment strategy. Conservation of the LSEC phenotype and prevention of capillarisation may reduce the risk of progression from inflammation and fibrosis to irreversible cirrhosis and HCC. However, this approach would require timely detection of disease to allow early intervention and prophylaxis. This may be clinically challenging, since liver disease is often initially silent, presenting only in patients with advanced fibrosis. Nevertheless, inhibition of LSEC capillarisation and dysfunction may impede the feed-forward impact on HSC activation, thus promoting fibrosis regression.

Angiogenesis in the context of fibrosis and tumourigenesis has been discussed in this review. The anti-inflammatory and anti-fibrotic effects of anti-angiogenic drugs is well-documented, with some candidates showing promise for CLD and cancer indications. These include peptibody, L1-10, which interferes with angiopoietin-2/Tie2 interactions, AT-II inhibition and multi-kinase inhibitors, sorafenib and lenvatinib, all of which have shown to inhibit both angiogenesis and tumour progression. Other potential targets include AEP and LECT2-Tie1 signalling, although further work is required to validate these candidates.

The recruitment of distinct leukocyte subsets by LSEC presents an opportunity for potential liver- and cell-specific therapeutic targets. Conventional adhesion molecules VCAM-1 and ICAM-1 are known to be upregulated in CLD and cancer, which facilitate leukocyte recruitment. Anti-inflammatory drug, resveratrol, inhibits VCAM-1 expression on tumour-activated LSEC and reduced hepatic melanoma metastases by 75% in a murine model (Salado et al., 2011). Furthermore, ICAM-1 has been demonstrated to play a role in all stages of the metastatic process from adhesion to immune evasion and colonisation. Therefore, ICAM-1 blocking antibodies might represent an avenue of therapeutic interest. To our knowledge, no group has successfully translated ICAM-1 blockade in clinical studies, owing to the challenges associated with inhibition of a cellular adhesion molecule with such important roles in homeostasis (Benedicto et al., 2017).

An alternative approach could be to target atypical adhesion molecules, which are also increased during inflammation and cancer, but display more organ-specific expression. These atypical adhesion molecules include scavenger receptors and VAP-1 which are detailed below. Pre-clinical studies suggest that these endothelial receptors may have roles in recruiting specific subsets of immune cells compared to the more global role of ICAM-1 and VCAM-1. For example, stabilin-1 has been shown to promote $T_{reg}$ recruitment (Shetty et al., 2011) and SCARF1 mediates adhesion of $CD4^+$ T cells rather than $CD8^+$ T cells (Patten et al., 2017a), under conditions of physiological shear stress, although their specific ligands on T cells are yet to be identified. Endothelial stabilin-1 expression localises to sites of leukocyte recruitment in inflamed liver and is also known to orchestrate B cell recruitment, facilitating adhesion and subsequent intravascular accumulation of B lymphoma cells (Shetty et al., 2012). Targeting of stabilin-1 may therefore allow modulation of the hepatic $T_{reg}$ pool or prevention of tumour metastasis; indeed, genetic deficiency or antibody blockade of stabilin-1 reduces immunosuppressive leukocytes within tumours and halts tumour progression in mice (Karikoski et al., 2014). SCARF1 has been shown to be upregulated in the hepatic sinusoids and at the sites of tissue fibrosis in a range of human CLDs (Patten et al., 2017a). Interestingly SCARF1 is also expressed in well-differentiated HCC tumours, whilst being downregulated in poorly differentiated HCC. VAP-1 was previously shown to promote the recruitment of Th2 lymphocytes rather than the Th1 subset in models of concanavalin A-induced liver injury (Bonder et al., 2005). VAP-1 is highly expressed in CLD (McNab et al., 1996; Weston et al., 2015) and mediates recruitment both directly (Lalor et al., 2002) and indirectly via its enzymatic activity, which can upregulate expression of other adhesion molecules (Lalor et al., 2007; Liaskou et al., 2011). VAP-1 is also expressed by HSCs; blockade of both adhesive and enzymatic properties of VAP-1 attenuates fibrosis, highlighting its therapeutic potential (Weston et al., 2015). Another receptor which has recently been highlighted in single-cell studies of the liver is PLVAP, which is an endothelial-specific protein known to be involved in leukocyte migration across lymphatics (Keuschnigg et al., 2009; Rantakari et al., 2015). Whilst it has an instrumental role in foetal liver-derived immune cell distribution (Rantakari et al., 2016), its involvement in leukocyte recruitment within adult liver remains to be explored. Nevertheless, PLVAP seems to be upregulated during inflammatory states (Ramachandran et al., 2019;

Su et al., 2020), and its re-emergence in the HCC microenvironment (Wang et al., 2014) suggests a role in pathogenesis which requires further investigation.

Inhibition of LSEC-cancer cell interactions represents an additional strategy. Blockade of L-SIGN has shown promise in reducing colon cancer metastasis in murine models (Zuo et al., 2013; Na et al., 2017), whilst MR has been suggested as a potential target for treating hepatic metastases and increasing anti-tumour cytotoxicity (Arteta et al., 2010). Moreover, LSEC Notch activation negatively regulates tumour cell adhesion in a Wnt-independent manner, thus protecting against liver metastasis (Wohlfeil et al., 2019). Selective modulation of Notch activity has been proposed as a therapeutic option in liver metastasis. The findings presented here suggest that the targeting of LSEC receptors and LSEC-specific pathways may be an attractive therapeutic strategy to modulate the hepatic immune microenvironment. Which pathway to target will be context-dependent; in CLD, the aim would be to promote anti-inflammatory and pro-resolution conditions, while in HCC, there will also be a need to drive an immunogenic, anti-tumoural environment to support cancer immunotherapy. Furthermore, targeting of cancer cell adhesion to LSEC may offer a route to mitigate frequent metastasis of solid tumours.

It is likely that the success of future LSEC treatments will depend on the reliable delivery of agents to the sinusoidal vascular bed in order to avoid systemic complications. This has led to several approaches to directly target LSEC in pre-clinical models (Poisson et al., 2017). For example, nanoparticles developed to interact exclusively with LSEC have been trialled in murine models of liver metastasis. Yu and colleagues reported that LSEC are specifically targeted by alpha-melittin nanoparticles (Yu et al., 2019), which increase numbers of innate and adaptive immune cells in addition to promoting T cell activation and NK cell maturation. Their ability to prime the anti-tumour response prevents the formation of metastasis. Moreover, melittin nanoparticles have a direct cytotoxic effect on metastatic tumours. Mice treated with melittin nanoparticles had significantly improved survival rates (Yu et al., 2019). Using miRNA conjugated with chondroitin sulfate-functionalised nanoparticles, Marquez and colleagues targeted LSEC activation during angiogenesis. MiR-20a, a molecule known to be downregulated in colorectal liver metastasis, was replaced in LSEC of murine models of liver metastases (Marquez et al., 2018). They found that targeting MiR-20a replacement reduced LSEC recruitment into metastatic foci, and metastasis size decreased by 80% following treatment. The development of nanoparticles specifically targeting LSEC is of particular interest as it enables a narrow therapeutic focus, potentially reducing the risk of systemic toxicity. These approaches will hopefully lead to the successful translation of LSEC-specific agents into clinical trials.

## CONCLUSION

LSEC play essential roles in physiology and homeostasis, with their dysfunction preceding fibrogenesis that yields high risk of carcinogenesis. Context-dependent LSEC signalling determines the outcome of liver injury, either tissue regeneration or fibrosis, providing strong support for LSEC as a target for fibrotic disease. Furthermore, their active involvement in modulating the immune and tumour microenvironment not only fosters primary and secondary tumour development, but also offers promising opportunities for therapeutic intervention.

## AUTHOR CONTRIBUTIONS

AW, MQ, and SS wrote the review. AW prepared the figures. All the authors contributed to the article and approved the submitted version.

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# Multiscale and Multimodal Optical Imaging of the Ultrastructure of Human Liver Biopsies

*Cihang Kong[1†], Stefanie Bobe[2†], Christian Pilger[1], Mario Lachetta[1], Cristina Ionica Øie[3], Nils Kirschnick[2], Viola Mönkemöller[1], Wolfgang Hübner[1,4], Christine Förster[4], Mark Schüttpelz[1], Friedemann Kiefer[2]*, Thomas Huser[1,4]* and Jan Schulte am Esch[4,5]**

[1] Department of Physics, Bielefeld University, Bielefeld, Germany, [2] European Institute for Molecular Imaging, University of Münster, Münster, Germany, [3] Vascular Biology Research Group, Department of Medical Biology, University of Tromsø – The Arctic University of Norway, Tromsø, Norway, [4] Forschungsverbund BioMedizin Bielefeld (FBMB), Bielefeld, Germany, [5] Department of General and Visceral Surgery, Evangelisches Klinikum Bethel gGmbH, University Hospital OWL of the University of Bielefeld, Bielefeld, Germany

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Alexei Tepikin,
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Medical University of Graz, Austria

***Correspondence:**
Friedemann Kiefer
fkiefer@uni-muenster.de
Thomas Huser
thomas.huser@physik.uni-bielefeld.de
Jan Schulte am Esch
jan.schulteamesch@evkb.de

[†] These authors have contributed equally to this work

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The liver as the largest organ in the human body is composed of a complex macroscopic and microscopic architecture that supports its indispensable function to maintain physiological homeostasis. Optical imaging of the human liver is particularly challenging because of the need to cover length scales across 7 orders of magnitude (from the centimeter scale to the nanometer scale) in order to fully assess the ultrastructure of the entire organ down to the subcellular scale and probe its physiological function. This task becomes even more challenging the deeper within the organ one hopes to image, because of the strong absorption and scattering of visible light by the liver. Here, we demonstrate how optical imaging methods utilizing highly specific fluorescent labels, as well as label-free optical methods can seamlessly cover this entire size range in excised, fixed human liver tissue and we exemplify this by reconstructing the biliary tree in three-dimensional space. Imaging of tissue beyond approximately 0.5 mm length requires optical clearing of the human liver. We present the successful use of optical projection tomography and light-sheet fluorescence microscopy to derive information about the liver architecture on the millimeter scale. The intermediate size range is covered using label-free structural and chemically sensitive methods, such as second harmonic generation and coherent anti-Stokes Raman scattering microscopy. Laser-scanning confocal microscopy extends the resolution to the nanoscale, allowing us to ultimately image individual liver sinusoidal endothelial cells and their fenestrations by super-resolution structured illumination microscopy. This allowed us to visualize the human hepatobiliary system in 3D down to the cellular level, which indicates that reticular biliary networks communicate with portal bile ducts via single or a few ductuli. Non-linear optical microscopy enabled us to identify fibrotic regions extending from the portal field to the parenchyma, along with microvesicular steatosis in liver biopsies from an older patient. Lastly, super-resolution microscopy allowed us to visualize and determine

the size distribution of fenestrations in human liver sinusoidal endothelial cells for the first time under aqueous conditions. Thus, this proof-of-concept study allows us to demonstrate, how, in combination, these techniques open up a new chapter in liver biopsy analysis.

**Keywords: liver biology, liver morphology, liver sinusoids, light-sheet fluorescence microscopy, coherent Raman scattering microscopy, super-resolution optical microscopy, liver sinusoidal endothelial cells.**

## INTRODUCTION

The human liver is the largest internal organ of the human body and indispensable for the maintenance of physiological homeostasis. Essential functions of the liver include uptake and metabolism of nutrients, synthesis of glycogen, lipids, amino acids and hormones, and the production and secretion of serum proteins including various lipoproteins, albumin and the constituents of the coagulation system. Fast access to dietary components and xenobiotics entering the circulation predestines the liver to a prime role in the uptake and storage of vitamins and metals, carbohydrate metabolism but also detoxification in particular of hydrophobic substances. The liver is the central metabolic hub of any organism and at the same time responsible for the production of bile and digestive factors and the discharge of metabolic end products and solubilized hydrophobic molecules.

The central physiological function and metabolic activity of the liver are reflected by its unique circulatory integration. About 75% of the blood supply to the liver are delivered by the portal vein providing rapid access to newly absorbed dietary constituents. The remaining perfusion is comprised of freshly oxygenated blood provided by the hepatic artery. Inflowing blood is split among the eight segments of the two liver lobes, with each segment possessing its independent vascular supply and extrinsic bile duct (**Figure 1A**).

Blood is rapidly distributed into smaller caliber vessels that end in hepatic capillaries of 5 to 10 μm diameter, termed sinusoidal vessels, which within a liver lobule drain into the same central vein (**Figures 1B,C**). Strands of hepatocytes, termed trabeculae, which at their apical junction form bile canaliculi, embed the sinusoidal vessels and together, hepatocytes and endothelial cells constitute the functional units of the liver. Sinusoidal liver endothelial cells (LSECs) are a highly specialized type of endothelium with unique morphology and functions (Sørensen et al., 2015). LSECs contain many small transmembrane pores, or fenestrations, with average diameters of 100 – 150 nm, within a range of 50 – 300 nm (Braet and Wisse, 2002; Øie et al., 2018), which provide open channels between the sinusoidal blood and the subendothelial space, the "Space of Disse," facilitating the transfer of substrate between the blood and the parenchymal hepatocytes (**Figure 1D**). A striking functional characteristic of the LSEC is its high endocytic capacity via membrane associated receptors. LSECs do not have a basement membrane. Instead, they face the matrix "Space of Disse," the abluminal border of which is formed by the basal surface of the surrounding hepatocytes (**Figure 1D**) (Treyer and Müsch, 2013). LSECs continuously filter plasma via their fenestrae into the Disse space, where it is probed and its components are metabolized by hepatocytes.

The Disse space also harbors hepatic stellate cells, which store, among other lipophilic factors, vitamin A, while the liver-resident macrophages, Kupffer cells, patrol the luminal side of the sinusoidal vessels (**Figure 1D**). The regular arrangement of the maximum possible number of liver lobules results in a classic honeycomb structure where each lobule is surrounded by six neighboring lobules, characterized by their centrally located veins and a portal field at each tripartite junction of the hexagonal edges (**Figures 1B,C**). In addition to the portal arteries and veins, portal fields also contain the hepatic lymphatic vessels that were reported to originate as cul-de-sacs.

The apical surface of the hepatocytes form the biliary canaliculi, which at the border to the portal fields merge into the bile duct as part of the portal triad. Liver pathologies directly impact on cholangiocyte structure and function, and may result in cholestasis causing inflammation and liver dysfunction. Conversely, cholangiocyte dysfunction may actively initiate or foster inflammation causing or contributing to liver injury. In particular, the 3-dimensional structure of the transitory zone between bile canaliculi and the more robust intrinsic bile ducts, also called Canal of Hering, has so far not been visualized in the human liver by volume imaging. This transitory zone between the liver parenchyma and the intrinsic biliary tree is partly lined by hepatocytes, partly by small cholangiocytes, and demonstrates the upregulation of EpCAM + hepatic stem/progenitor cells in scenarios of large scale hepatocellular injury with the subsequent ductular reaction (Dollé et al., 2015). Given the important implication of this area in case of dysfunction for the development of liver disease but also for liver regeneration following high-grade liver damage, a detailed structural understanding is highly warranted. Here, we demonstrate how the high level of specificity as well as the deep penetration depth of fluorescent and label-free optical microscopies allow us to image these liver structures across 7 orders of magnitude - from the centimeter scale down to the nanoscale.

## MATERIALS AND METHODS

### Harvesting of Human Liver Biopsy Samples

Informed consent according to local and international guidelines was signed by all patients. All further experimental procedures were ethically approved (Ethics committee Münster, Germany,

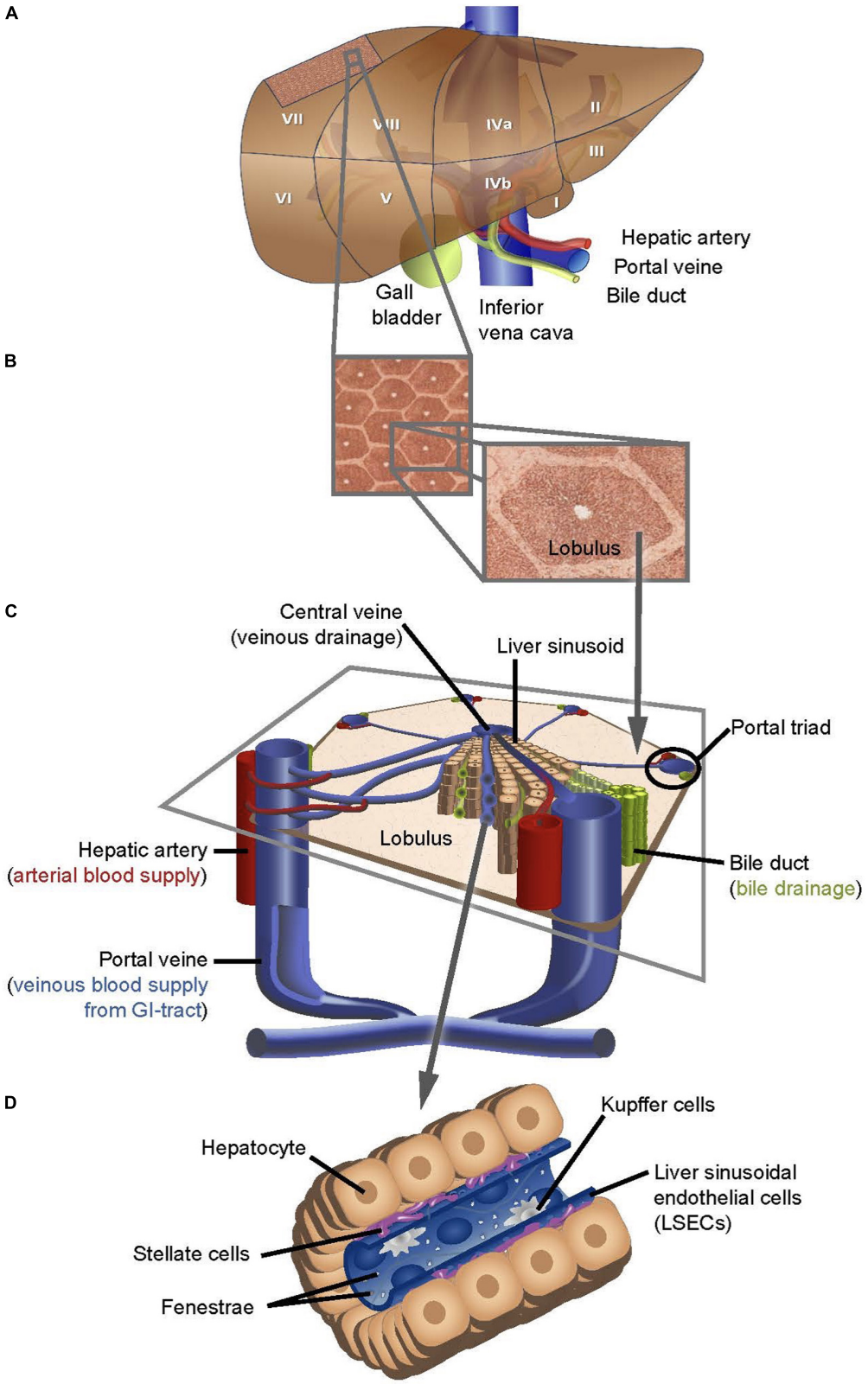


**FIGURE 1 |** Schematic depiction of the structure of the human liver at different scales of resolution. **(A)** Traditional segmentation used in anatomy and surgery, subdivides the human liver into eight segments. **(B)** Each segment is composed of numerous liver lobules that are packed in a honeycomb pattern (**B**. left panel) and individual lobules are separated by bands rich in extracellular matrix (**B**. right panel). **(C)** Every tripartite junction between liver lobules forms a portal field, composed

*(Continued)*

**FIGURE 1 |** Continued
of a venous vessel originating from a branch of the portal vein, an arterial vessel originating from the hepatic artery and one or more bile ducts. The basic functional unit of the liver is comprised of a central sinusoid flanked by trabeculae of hepatocytes that enclose with their apical membrane a primary bile canaliculus, finally draining into the bile duct in the portal triad. All sinusoids of a lobule drain into a single central venous vessel. **(D)** The distance between the fenestrated endothelial cells that form the sinusoids and the hepatocyte canaliculi is referred to as Space of Disse and contains stellate cells, while Kupffer cells patrol within the endothelial lumen.

2017-522-f-S). Liver samples of a size of roughly 1 $cm^3$ were obtained from a human patient suffering from a hepatically metastasized rectal adeno carcinoma following robotic assisted deep anterior rectal resection 18 months prior to the actual hepatic resection of metachron liver metastases in hepatic segments III and IVa. The liver resection was performed subsequent to an induction of chemotherapy according to the FOLFOXIRI-protocol (11 cycles) with a systemic therapy-free interval of six weeks. Following tissue sampling for routine histopathological purposes, study samples were harvested from a non-neoplasm-involved area of the resected tissue sample (segment III) by an experienced pathologist. Classical hepatic histopathology of the here utilized non-tumor affected liver-tissue revealed no significant pathology. Samples were cut from resected liver tissue and immediately placed in 4% formaldehyde for 1 hour at room temperature for fixation. They were subsequently transferred to a phosphate buffered saline solution containing 0.5% paraformaldehyde for longer term storage followed by specialized sample treatment as required for each specific imaging technique.

## Whole Mount Staining of Human Liver

Human liver tissue was fixed in 4% formaldehyde in phosphate buffered saline (PBS) for 2 h, permeabilized (5% Triton X-100/PBS) and subsequently blocked in Permblock solution (3% BSA, 0.5% Tween-20 in PBS). For whole mount immunostaining anti-cytokeratin 19 (proteintech, number 14965-1-AP), anti-αSMA-Cy3 (Sigma, clone 1A4) and Alexa647-coupled secondary antibody (Molecular Probes) were used in Permblock solution. Antibody incubation was performed for at least 3 weeks at 37°C and samples were washed with PBS-T (0.1% Tween-20/PBS) after each step. The whole mount stained samples were embedded in cylindrical 1% low melting agarose to avoid light scattering at agarose edges during later imaging. Following dehydration and delipidation in increasingly concentrated methanol (70%, 95%, > 99%, > 99%, each step at least 2 h), optical clearing was performed by gradually replacing methanol with a 1:2 benzyl alcohol-benzyl benzoate solution (BABB, Murray's clear) for refractive index matching. Samples were equilibrated in BABB at least one day and subsequently imaged by light sheet fluorescence microscopy (LSFM) and optical projection tomography (OPT).

## Preparation of Liver Sections for Non-linear Optical Imaging

In order to accommodate the forward-scattering geometry of non-linear optical microscopy methods, such as coherent Raman scattering and second harmonic generation microscopy, where the excitation light is focused into the sample on an inverted microscope and the portion of the light converted to another wavelength within the sample is collected in the forward direction, 50 μm thick sections of liver samples were prepared. Freshly excised liver cubes of approx. 1 $cm^3$ volume were stored on ice and were then embedded in TissueTek O.C.T. compound prior to cryo-microtome sectioning. After freezing the tissue blocks were cut to 50 μm thick slices, which were then placed on a glass coverslip (#1, Roth, Karlsruhe). After fixation in 4% formaldehyde for 30 min at RT samples were rinsed with 0.5% formaldehyde. A second cover slip was then placed on top and forms a sandwich around the liver sample for the measurements.

## Isolation and Staining of Human Liver Sinusoidal Endothelial Cells (LSEC)

Liver tissues were obtained from patients undergoing hepatic resections for liver metastasis from colorectal carcinoma. Ethical approval for the study was granted by the Norwegian Research Ethics Committee, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each patient. Human LSECs were isolated based on a method developed at the Vascular Biology Research Group, UiT-The Arctic University of Norway, Tromsø, using Percoll gradient and magnetic-activated bead cell sorting (Øie et al. - manuscript in preparation). The cells were seeded on fibronectin coated 13 mm #1.5 glass bottom dishes (MatTek, Ashland, MA, United States) at a density of $3 \times 10^4$ cells/$cm^2$. Following attachment and spreading of the cytoplasm, the cells were fixed in 4% formaldehyde, stained and imaged. To visualize fenestrations, the plasma membrane was stained for 10 min with CellMask Orange (1:1000 in PBS).

## Optical Projection Tomography

An optical projection tomography system was built following the description by Nguyen et al. (2017). In essence, this system resembles a widefield-fluorescence microscope using long focal length achromatic lenses for excitation and detection and utilizes a pinhole to extend the depth of field across the sample. Samples as large as 27 mm × 27 mm × 27 mm can be imaged with a spatial resolution down to 30 μm, enabling the examination of entire rodent organs. Samples were rotated within the focus of the system by 360° and images are taken with a step size of 1° or less. To penetrate through the entire sample, optical clearing of the samples is required, though. The only major modification to Nguyen et al. was the use of a different camera. Here, a CMOS camera with 20 MPixels and a pixel size of 2.4 μm was used (Tucsen FL-20BW). For excitation, LED light sources and corresponding filter sets were used as follows: For Alexa488, a filter set containing a 480/30 nm bandpass filter as excitation filter, a 505DC dichroic mirror, and a combination of a 535/50 nm and 520/40 nm bandpass filters as emission filters,

for Alexa647, a filter set containing a 625/20 nm bandpass filter as excitation filter, a 650DC dichroic mirror, and a 700/75 nm bandpass filter as emission filter, for Cy3, a filter set containing a 550/60 nm bandpass filter as excitation filter, a 588DC dichroic mirror, and a combination of bandpass and longpass filters (545/30 nm, 593/40 nm bandpass filter in conjunction with a 568 nm longpass filter as detection filter. The system provides a magnification factor of 0.5 with an aperture set to 12 mm diameter providing a depth of focus of approx. 2 mm. For the OPT images shown here, 600 images at a rotation step size of 0.6° were acquired for each color channel. The resulting raw images were reconstructed using a filtered-back projection (FBP) algorithm utilizing a Shepp-Logan filter (Vallejo Ramirez et al., 2019) (reconstruction software: [1]).

## Light Sheet Fluorescence Microscopy (LSFM)

After whole mount immunostaining and optical clearing, liver tissue was imaged using a LaVision UltraMicroscope II (LaVision BioTec) with a step size of 2 μm and either at 1.6-fold or 5-fold magnification. 3D reconstructions of the acquired stacks were visualized and analyzed using the volume rendering software package Voreen (*voreen.uni-muenster.de*) (Meyer-Spradow et al., 2009; Hägerling et al., 2017).

## Non-linear Optical Microscopy (SRS, CARS, SHG)

A custom-built coherent Raman scattering microscope was used for label-free imaging of liver sections. The laser source consisted of a 1032 nm fiber laser (Emerald Engine, APE, Berlin) operating at 80MHz repetition rate with 2 ps pulse length. The frequency-doubled 516 nm beam pumped an optical parametric oscillator (OPO) (Levante Emerald, APE, Germany), producing a beam with tunable wavelength, which was utilized as pump beam in the coherent anti-Stokes Raman scattering (CARS) and stimulated Raman scattering (SRS) experiments. The 1032 nm laser served as non-tunable Stokes wavelength. The pulsed pump and Stokes laser beams were overlapped in time and space by a dichroic mirror and an optical delay stage. The combined beams are then sent into a custom-built laser scanning microscope. Galvanometric scanning mirrors (Cambridge Technology, Galvanometer Optical Scanner, Model 6215H, United States) were utilized to raster scan the laser focus across the sample. A scanning telescope filled the back focal plane of a 60x water immersion objective lens (Olympus UPlanSApo, NA1.2, Olympus, Germany) focusing the beams into the sample. The signal generated in the sample was collected in the forward direction using an oil immersion condenser lens (U-AAC, NA1.4, Olympus, Germany). The CARS signal was isolated from the excitation laser wavelengths by an optical filter set composed of a 950 SP (short pass), a 785 SP, two 775 SP, and a 514 LP (long pass) filter (all Semrock, United States) and a 650/40 BP (bandpass) filter. Detection of the CARS signal is accomplished by a photomultiplier tube (PMT (H 9656-20, Hamamatsu Photonics, Japan). The resulting electronic signal was acquired by an analog-to-digital (A/D) converter (PCI-6110S, National Instruments, United States) and used for visualization by the MATLAB program ScanImage (version 3.8.1, Howard Hughes Janelia Farm Research Campus). For SRS imaging the Stokes beam was modulated by a resonant electro-optic modulator operating at 20 MHz. For the acquisition of the SRS signal the pump beam was isolated from all other wavelengths by the 950 SP and an 800/50 BP (Chroma) filters and was directed onto a customized photodiode. The electronic signal was filtered and demodulated in combination with the 20 MHz reference signal by a specifically adapted lock-in amplifier (APE, Berlin), which couples the demodulated SRS signal into the A/D converter card. For CARS imaging the 2845 $cm^{-1}$ molecular resonance was probed to visualize the lipid distribution in the sample. The focal intensities were set for the Stokes beam (1032 nm) at 16.5 mW and for the pump beam (799.3 nm) at 33 mW, respectively. For second harmonic generation (SHG) imaging the laser source was switched to a home-built fiber-based femtosecond laser with 400 fs pulse length (55MHz repetition rate) (described in Kong et al., 2017) operating at 1054 nm using 20 mW focal intensity while the filter set was changed to a bandpass at 532/18 nm and the 950 SP as well as two times the 785 SP to isolate the SHG signal.

## Confocal Microscopy

In preparation for confocal microscopy, human liver tissue was fixed in 4% formaldehyde/PBS for 2 h, embedded in 6% low melting point agarose and 100 μm thick vibratome sections were prepared. The sections were subsequently permeabilized (0.5% Triton X-100/PBS) and blocked in Permblock solution (3% BSA, 0.5% Tween-20 in PBS). Antibodies used have been described under whole mount preparations. Sections were incubated overnight at 4°C in primary antibody dilution, washed three times with PBS-T (0.1% Tween-20/PBS) and finally incubated in secondary antibodies for 1 h at room temperature. The stained samples were mounted with Mowiol and imaged using a commercial laser scanning fluorescence microscope (Zeiss LSM880, 20x, NA = 0.8).

## Super-Resolution Structured Illumination Microscopy (SR-SIM)

Human LSEC were imaged using a commercial super-resolution structured illumination microscope (SR-SIM) (DeltaVision| OMXv4.0 BLAZE, GE Healthcare) equipped with a 60 × 1.42NA oil-immersion objective lens (Olympus). 3D SR-SIM image stacks of 1 μm thickness were acquired with a vertical distance between image planes of 125 nm and with 15 raw images per plane (five phases, three angles). Raw datasets were computationally reconstructed using the SoftWoRx software (GE Healthcare). For clarity of display, figure images were linearly adjusted for brightness and contrast using Fiji ([2]version 2.0.2.) (Schindelin et al., 2012).

[1]https://lag-opt.github.io/

[2]https://fiji.sc

## RESULTS AND DISCUSSION

Optical imaging methods offer a highly "natural" way of analyzing tissue samples because they extend and exploit the evolutionarily optimized strong human visual perception and have therefore been employed by scientists for centuries. The combination of particularly gentle preparation techniques (in comparison to the sample preparation required for electron-microscopy) with label–free imaging or genetically encoded reporters, such as fluorescent proteins, allow the application of optical methods under conditions that preserve the sample in its most natural state, where even mechanical and morphological sample properties such as elasticity, size and shape are mostly maintained. It should be mentioned that this statement is no longer correct if fixation, permblock or dehydration and refractive index matching are used as part of the sample preparation, in which case control experiments need to be considered in order to ensure that sample preparation does not interfere with the conclusions drawn from optical measurements. A particular strength of optical imaging modalities is, however, that they benefit from a wide range of contrast methods that have been developed, in particular during the last couple of decades. Fluorescence microscopy is an especially attractive method, because fluorescent staining of samples provides highly specific molecular contrast. This is achieved either through the use of organic fluorophores, which bind specific molecular structures within the sample (e.g., lipophilic dyes will stain lipids, intercalating dyes will stain chromatin or nucleic acids, etc.) or through incubation of the sample with highly specific binders such as antibodies or nanobodies to which fluorophores have been coupled. An approach intensely developed in the recent past is the expression of molecular tags with the capacity to bind fluorophores or to convert non-fluorescent dyes to a fluorescent form. In the following, we demonstrate how excised and fixed samples of the human liver can be visualized and analyzed by optical microscopy methods from the millimeter scale down to the nanometer scale. We provide examples for mesoscale, microscale and nanoscale fluorescence microscopy methods and their partial combination that allow for comprehensively imaging liver morphology. In addition, we demonstrate how these methods can be further enhanced by the introduction of highly specific label-free chemical imaging techniques.

### Mesoscopic Imaging of the Human Liver by Fluorescence Microscopy

The scale on which a sample can be imaged and the spatial resolution, which is achieved by a particular method typically go hand-in-hand - at least if the acquisition times for the imaging process shall be kept reasonable. Thus, for imaging liver tissue on the millimeter scale, the spatial resolution is typically limited to the micron scale. A particularly attractive method for imaging entire millimeter sized liver samples that was developed within the last 2 decades is optical projection tomography (Sharpe et al., 2002). Optical projection tomography (OPT) is the optical analog to X-ray computed tomography. In OPT light is passed through a sample and an image is taken with a camera. The sample is then rotated in the light path at small inclinations and additional images are taken at every step until the sample was rotated by 180°, or better 360°. The images that were collected in this way could be either transmitted light images or fluorescence images and each pixel in an image represents the line integral of the chosen contrast projected through the sample. Three-dimensional images of the sample are then reconstructed using a filtered back-projection algorithm based on an inverse Radon transform of the data (Sharpe, 2004). This method does, however, require that light can be transmitted without significant absorption or scattering through the entire sample, which is rather difficult to achieve in the case of the liver. Thus, optical clearing methods are typically applied to generate optically transparent samples, with protocols based on organic solvents having the longest history. Organic solvents extract lipids from the sample and hydrate the tissue. In a subsequent step the water in the sample is replaced by a refractory index matching liquid, such that structures that lead to the absorption or scattering of light are largely removed or minimized (Orlich and Kiefer, 2018). The result of this is shown in **Figure 2**, where two liver biopsy specimens obtained from the same human donor were immunostained, subsequently optically cleared and then imaged by OPT. A representative photograph of one of these optically cleared liver samples is shown in the inset in the upper right corner of row A in **Figure 2**. Samples were whole mount immunostained for a smooth muscle actin (aSMA) and the intermediate filament cytokeratin 19. aSMA decorates smooth muscle cells, which are present as mural cells in the blood vessel walls, not notably in arteries and arterioles (shown as magenta in **Figure 2**). Cytokeratin 19, on the other hand, stains cholangiocytes, which results in highlighting the bile ducts (shown as white in **Figure 2**). As described in Materials and Methods, following delipidation and dehydration and refractive index matching in Murray's clear (BABB), samples were mounted on a rotation stage and fully immersed in Murray's clear within an imaging quality quartz cuvette for the imaging process. Fluorescence images were acquired sequentially for the different fluorophores.

Each of the samples in **Figures 2A,B** covers a volume of approximately 3 × 3 × 5 mm. A movie showing the 3D reconstruction of the OPT data is presented in **Supplementary Video 1**. Center panels in **Figure 2** show projections of the fluorescence from the entire sample, left panels show optical cross sections at the level indicated by the blue dashed lines while the right panels depict projections of a stack of optical cross sections indicated by the yellow dashed rectangles. The aSMA staining revealed the distribution of liver arteries and veins, while the bile ducts were identified by cytokeratin. We noted a relatively high background of the aSMA staining, depicted in the magenta channel, which was augmented by intense aSMA signal from arteries and arterioles throughout the specimen. This effect becomes naturally most obstructive in the maximum intensity projections shown in the central panels. In order to obtain a more detailed, higher resolution volumetric view, the same samples were also imaged by light sheet fluorescence microscopy (LSFM). Over the last decade light sheet microscopy, originally perceived by Siedentopf and Zsigmondy (1902) more than a century ago,

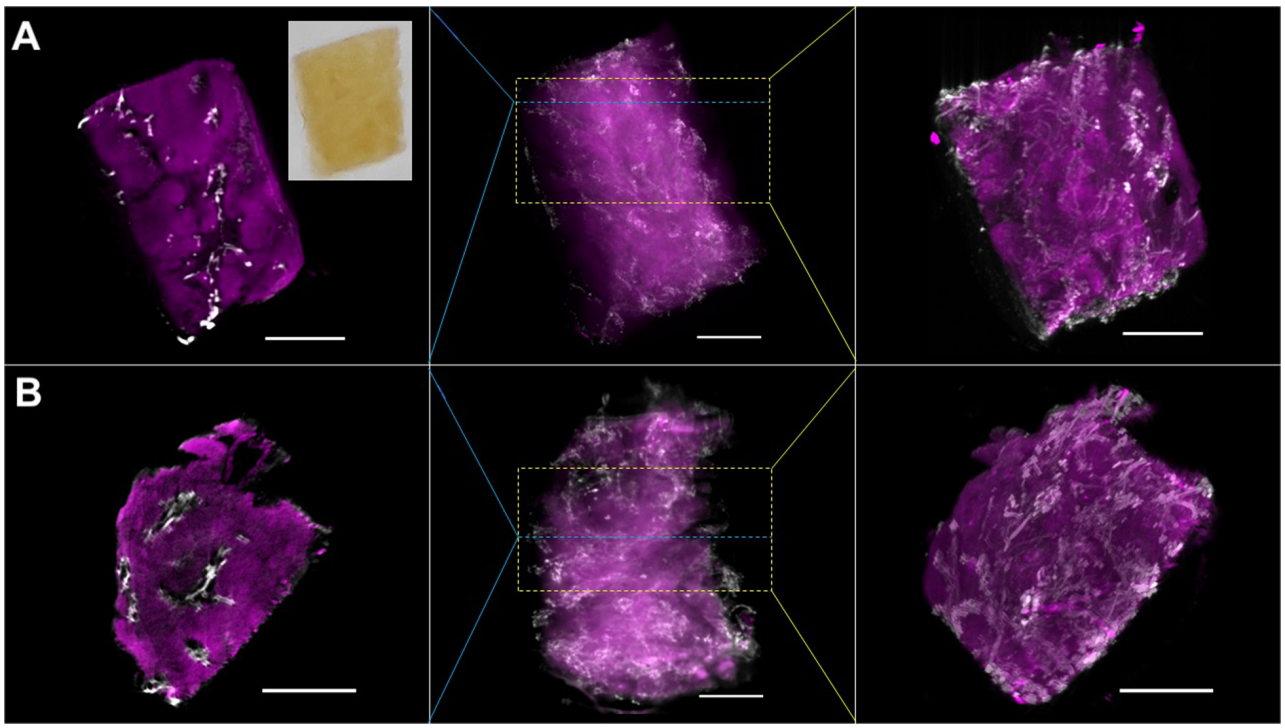


**FIGURE 2 |** Optical projection tomographs (OPT) of the human liver. **(A)** and **(B)** are two different liver biopsies obtained from the same patient, each covering a volume of approximately 3 mm × 3 mm × 5 mm. The images in the center column panels are projections of the fluorescence from the entire sample. The images in the left panels show single cross sections that were taken at the regions indicated by the blue dashed lines in each row. The images in the right panels are projections of a stack of cross sections, the extent of which is indicated by the yellow dashed box. The specimen were stained with antibodies against a smooth muscle actin identifying smooth muscle cells (magenta, indicated by arrowheads in the individual panels) and cytokeratin 19 identifying bile ducts (white, indicated by asterisks in the panels) and subsequently cleared following the BABB protocol. The inset in the upper left panel shows a photograph of the optically cleared liver sample in BABB. Scale bars are 1 mm.

has undergone a vivid renaissance, which was originally sparked by the work of Stelzer and coworkers (Huisken et al., 2004). In contrast to the traditional fluorescence microscopy modalities such as epifluorescence, confocal and multiphoton microscopy, in LSFM the sample is not illuminated through the objective lens but orthogonally to the detection path by a thin sheet of light (Power and Huisken, 2017). This thin light sheet, generated by a scanned laser beam or more traditionally through shaping of a Gaussian beam using cylindrical lenses, is then exploited to scan the *z* axis of the sample in a stepwise fashion. Uncoupling of the illumination and detection light paths in this modality offers significant benefits. First, because fluorescence within the sample will only be excited in the volume illuminated by the light sheet, the thickness of the sheet defines the focal planar volume and hence z axis resolution. Therefore, further measures to suppress undesired out-of-focus fluorescence are not required. Second, because the sheet forming optics, which can be freely configured, defines the resolution in z, the lateral resolution of the detection optics can be matched to the sheet such that the resulting point spread function is close to isotropic. Third, the entire focal plane can be imaged simultaneously using an area detector (scientific camera), making image acquisition in light sheet microscopy significantly faster as e.g., scanning modalities such as confocal microscopy that rely on photomultipliers. Fourth, because only the actual volume being detected is illuminated, LSFM does not require illumination throughout the entire sample, which significantly reduces phototoxicity in live imaging and photobleaching of fixed, stained samples (Mertz, 2011).

While originally conceived for the analysis of colloidal dispersions in glass, an obvious limitation of LSFM is the requirement for nearly complete tissue transparency, which presently limits the technology to either transparent small model organisms such as zebrafish larvae or tissue samples that have undergone optical clearing. The roots of tissue clearing also date back longer than a century. A plethora of new and innovative approaches have been developed and refined over the last decade, described in a number of excellent recent reviews on the topic (Costantini et al., 2019; Matryba et al., 2019).

The results reported here were obtained with a basic single objective configuration, illuminated by a dual sided light sheet and we visualized image stacks using our proprietary volume-rendering framework Voreen (Meyer-Spradow et al., 2009; Dierkes et al., 2018). We analyzed the same immunostained liver biopsy specimen with LSFM that was later also imaged using OPT. A movie showing the 3D reconstruction of the LSFM data is shown in **Supplementary Video 2**. **Figure 3** shows a direct comparison of two corresponding planes of this sample imaged by OPT (**Figure 3A**) and LSFM (**Figure 3B**). Both technologies

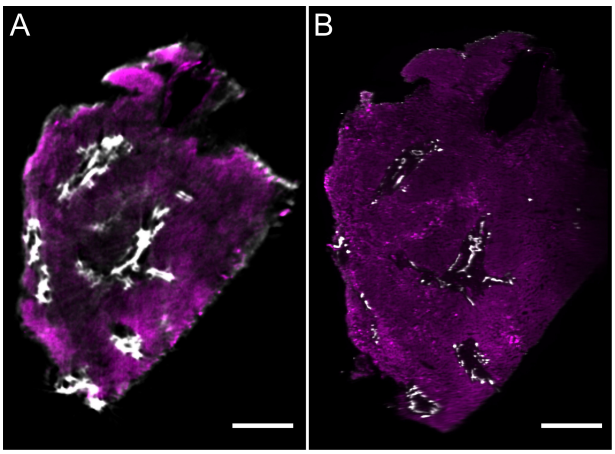


**FIGURE 3 |** Comparison of liver volumes imaged by optical projection tomography **(A)** and light sheet microscopy **(B)**. Optical sections of the same wholemount immunostained liver biopsy (for antibodies and colors also see **Figures 2**, **4**) were acquired with both optical projection tomography **(A)** and light sheet microscopy **(B)**. Image stacks were visualized using the volume rendering software package Voreen. Subsequently, rendered volumes were digitally oriented such that the sectional planes were approximately matching and virtually isolated, thin optical section corresponding to 9.6 μm **(A)** and 5 μm **(B)** are shown for direct comparison. Arrowheads identify corresponding structures. Scale bars represent 500 μm.

assess mesoscopic tissue volumes, hence corresponding tissue planes were identified *in silico* from the digitally 3D rendered volume representations. As can be seen from this comparison, both techniques provided an excellent representation of the bile ducts but also identified blood vessels in the surrounding tissue, albeit the staining contrast over background was less pronounced for these structures. As expected, OPT provided a somewhat lower spatial resolution, however, the specimen only occupied a fraction of the maximum imaging volume that can be assessed with this instrument. By adjusting the magnification of the lenses used in the OPT, the resolution could be matched to that of LSFM, but the main purpose of the instrument in its current state is to provide volumetric imaging of tissues that are typically too large to be imaged by LSFM, where the thickness to which a sample can be imaged depends mostly on the working distance of the objective lenses.

A particular strength of volumetric imaging of whole mount stained samples by OPT and LSFM is the minimized risk that rare events go unnoticed, which is a permanent danger in section-based approaches. Quantitative analysis based on this type of volume imaging usually gains enormously in statistical power over section-based analysis because the frequency of analyzed events is significantly increased. This is demonstrated in **Supplementary Video 1** for OPT data and **Supplementary Video 2** for LSFM data. In addition, the possibility to digitally reorient the sample freely on a personal computer is invaluable during structural and anatomical analysis. This is demonstrated in **Figure 4**, where portal regions can be effortlessly inspected in a cross sectional and a longitudinal view. In the latter, we were able to identify the affiliation of the blood vasculature with the arterial or portal venous tree based on the smooth muscle cell orientation (see **Figure 4B**, white arrowheads). The 3-dimensional structure of the smallest bile ducts originating at the hepatocyte canaliculi (Ducts of Hering) unexpectedly revealed a reticular network, often originating with bulbous small ductal stubs. Patches of this network then communicate via a single or few ductuli with the portal ducts.

## Microscopic Imaging of the Human Liver by Fluorescence and Label-Free Confocal Optical Microscopy

The large overview images provided by previously described mesoscopic imaging techniques make the identification of particular structures of interest straightforward. This is illustrated in the LSFM volume in **Figure 5A** where a portal region can readily be identified. Closer inspection at higher resolution then reveals the hepatocyte trabeculae of the adjacent lobule blood vessels (shown in magenta) and bile ducts (shown in white, see **Figure 5B**). The spatial resolution of LSFM is sufficient to identify the lumen even in the small Canal of Hering at the edge of the portal field and to discern individual cholangiocytes in the bile ducts. Confocal microscopy (Pawley, 2006), which for one-photon excited fluorescence is limited to sections of at most 100 – 200 μm thickness and small fields of view in the absence of image tiling, clearly surpasses the resolution achieved by mesoscale LSFM and allows us to reveal subcellular structures of the cholangiocytes and the fibers of the portal field extracellular matrix (ECM, see **Figure 5C**). This level of resolution is well complemented by non-linear optical imaging methods, such as multi-photon excited fluorescence, coherent Raman scattering, or higher harmonics generation within the sample, which, because these techniques utilize short laser pulses with wavelengths in the deep red to near infrared spectral region, can penetrate even deeper into the sample. This extended penetration depth is attributed to a lower absorption and less scattering of light within this spectral region in tissue.

Raman scattering, i.e., the inelastic scattering of photons by molecular bonds is a particularly interesting alternative to fluorescence excitation as it provides intrinsic, label-free chemical contrast of biological samples (Huser and Chan, 2015). Spontaneous Raman scattering by molecular bonds is a rather weak process due to its inherently low scattering cross section, but it can be significantly enhanced by coherent Raman scattering. Here, highly focused, short laser pulses specifically probe molecular vibrations of interest by a pump-probe type process. A pump photon initially prepares the samples for Raman scattering. Molecular bonds in the sample then interact with a simultaneously arriving probe photon in a process called four-wave mixing if the wavelength difference between the probe photon and the pump photon corresponds to the molecular vibration of interest. This interaction results in the emission of either a blue-shifted anti-Stokes photon in a process called coherent anti-Stokes Raman scattering (CARS) or in the emission of an additional photon with the same wavelength as the pump or the probe photon (stimulated Raman scattering, SRS). Over the

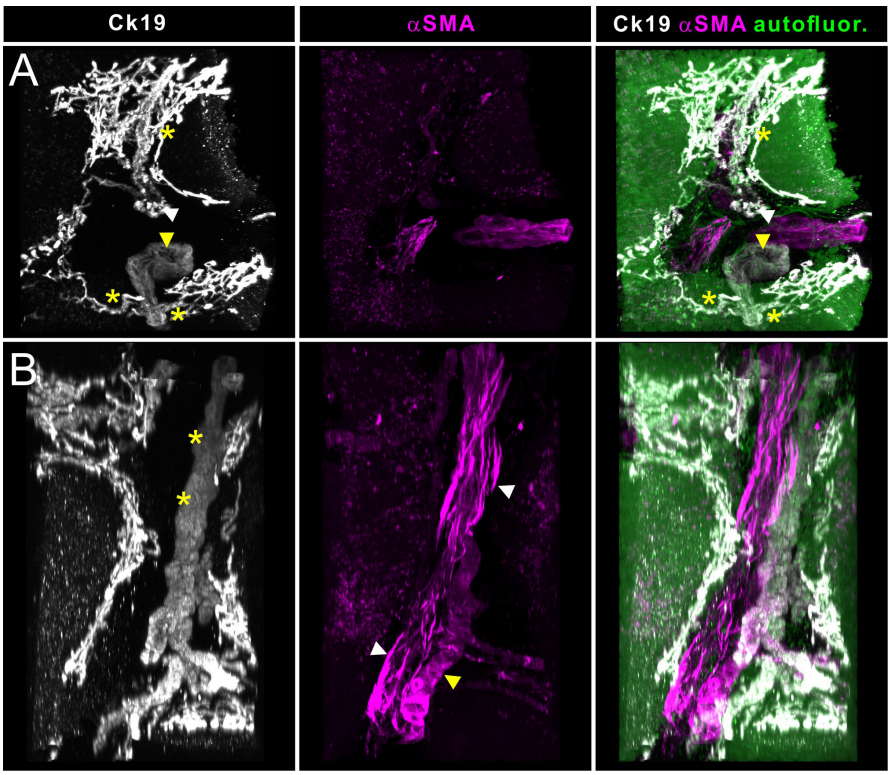


**FIGURE 4 |** Light sheet fluorescence microscopic analysis of human liver biopsies. Whole mount human liver biopsies stained for smooth muscle actin (magenta) and cytokeratin 19 (white) – identical specimen as depicted in **Figures 2**, **3**. Tissue autofluoresence (green) provided anatomical landmarks. Shown are maximum intensity projections of a tissue volume of approx. 1500 μm × 1300 μm × 800 μm. **(A)** Cross sectional aspect of a hepatic portal field. Besides the supplying blood vessels, shown in magenta in the central panel (likely parts of the portal venous connection), a smaller (white arrowhead) and a larger bile duct (yellow arrowhead) and the reticular network formed by their upstream smallest bile ductuli (Ducts of Hering). Communicating ducts between this reticular networks and the portal ducts are marked by asterisks. **(B)** Volume rendering showing a longitudinal aspect of the vasculature and bile ducts running in a portal field. Note the presence of two supplying blood vessels, which show distinct differences in the orientation of the smooth muscle cells in the vessel wall (central panel, magenta). Longitudinally running smooth muscle cells identify branches of the venous vasculature (white arrowheads), while a circumferential orientation of the smooth muscle cells is indicative of arterial vessels (yellow arrowheads). Cytokeratin staining is gradually downregulated in the more differentiated cholangiocytes of the larger caliber bile ducts (asterisk).

last decade, this method has gained considerable interest in the biomedical sciences and is now frequently used to image e.g., lipid deposits in tissues *in vitro* and *in vivo*, and even to generate virtual H&E staining contrast for *in vivo* pathology (Cheng and Xie, 2015). Here, we demonstrate how this process can be used for the analysis of human liver tissue on the scale of hundreds of microns. By combining this chemically specific imaging methodology with second harmonic generation (SHG), initiated by femtosecond laser pulses, contrast for fibrous structures can also be gained, further enhancing the range of label-free contrast methods. Due to the non-linear nature of the signal generation, CARS/SRS and SHG are confined to the focal region of the laser beams, resulting in a typical spatial resolution of $< 400$ nm and excellent optical sectioning capabilities in the axial direction in the range of $> 600$ nm. Thus, this intrinsic confinement of the signal generation permits three-dimensional imaging with little to no background signal. **Figure 6A** shows a large area scan of a liver tissue section with 40 μm thickness, where CARS was used as contrast mechanism. Here, the 2845 $cm^{-1}$ $CH_2$ stretching mode, which is predominantly associated with aliphatic lipid vibrations, is probed. By utilizing simple signal thresholding, the signal contribution is divided into signals below the threshold value (shown in magenta), which are typically due to a CARS-inherent non-resonant background and possibly lower lipid content in membranes and proteins. This signal enables us to visualize hepatocytes (**Figure 6A**), revealing also the position of their nuclei (blue arrow), and the sinusoids in between. The higher signal contribution above the threshold value is shown in yellow and allows us to identify lipid droplets (red arrow, **Figure 6A**) within the tissue. Subsequently, SHG imaging is performed as an additional contrast mechanism applied to the same sample area and overlaid in green, highlighting collagen structures due to the frequency doubling of a femtosecond fiber laser source. This contrast reveals fibrotic tissue sections within the liver tissue. In order to obtain an even larger field of view, several CARS images were acquired by automatic sample movement using a motorized stage and subsequent stitching of the individual images to obtain the large area view shown in **Figure 6B**. Here, the CARS image (still probing the $CH_2$ lipid resonance) reveals a portal vein with erythrocytes attached to the vessel wall (yellow arrow). In the

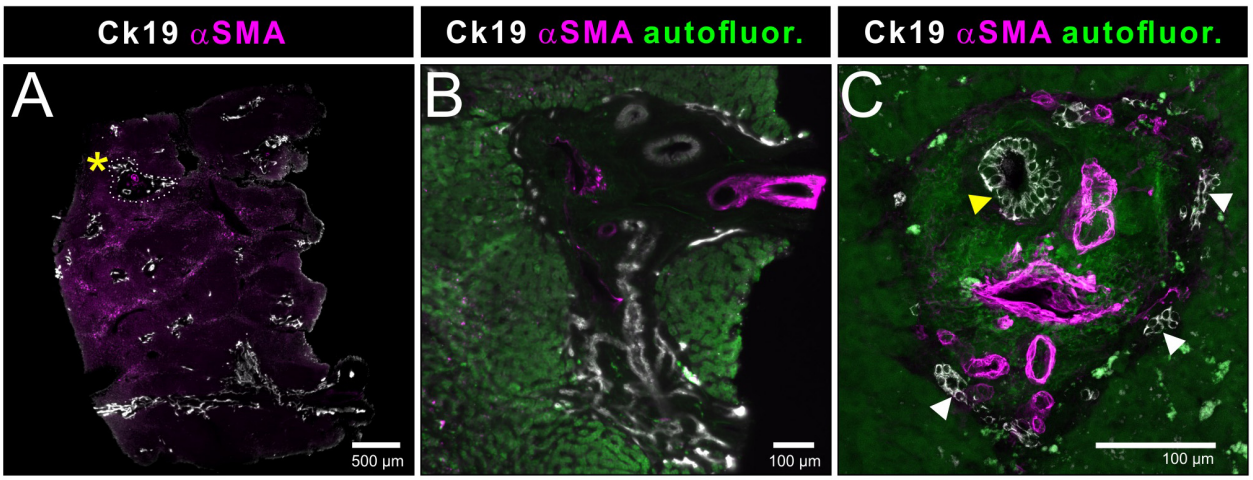


**FIGURE 5 |** Light sheet and confocal imaging of two representative human liver portal fields. **(A)** Light sheet microscopic overview of an immunostained human liver biopsy, in which specific structures of interest, here a prototypic portal field, are easily identified (encircled area next to asterisk). **(B)** Magnification of the cross section of one portal field from the rendered volume shown in **(A)**. **(C)** Confocal image of a portal field from a 100 µm section identically stained to the specimen depicted in **Figures 2**, **4**. Note the size difference of the cholangiocytes between the smallest bile ducts (white arrowheads) and the larger duct but also within the section of the larger bile duct (yellow arrowhead).

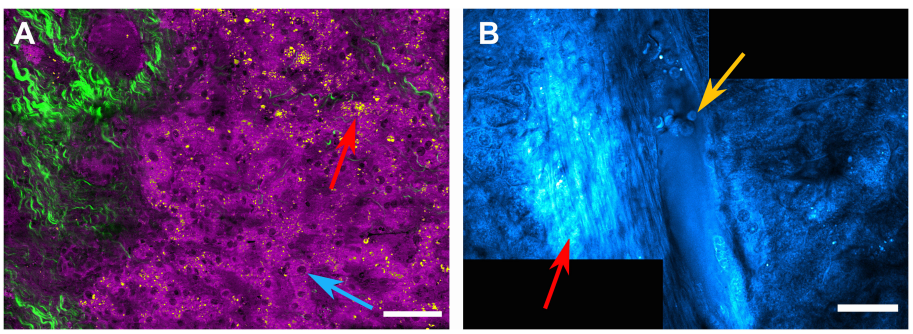


**FIGURE 6 |** Non-linear optical confocal microscopy of human liver biopsies. **(A)** CARS image probing the 2845cm$^{-1}$ lipid resonance. By signal thresholding, weak background signals depicting single hepatocytes and their nuclei (blue arrow) are shown in magenta and can be separated from lipid droplets, which provide signal above the threshold value (red arrow), shown in yellow. The green color depicts parts of the sample producing a SHG signal, which was acquired subsequently utilizing a femtosecond fiber-laser source. SHG indicates fibrous structures within the liver tissue. Scale bar is 50 µm - note that several images are stitched together to obtain a larger field of view. **(B)** CARS image (at 2845cm$^{-1}$) of a portal vein with erythrocytes attached to the lumen (yellow arrow). In the vicinity of the vein, fibrotic alterations of the tissue can be seen (red arrow). Scale bar is 30 µm.

vicinity of the vein, fibrotic tissue can, again, be seen (red arrow, **Figure 6B**).

To further improve the separation of nuclei and other cellular components, a more sophisticated method, called hyperspectral imaging, can be applied to sample areas of interest previously identified by the faster single resonance CARS microscopy (Cheng and Xie, 2015; Pilger et al., 2018). In hyperspectral imaging, not only a single molecular resonance is probed, but resonances within an entire wavelength range are acquired by automated wavelength tuning of the pump beam source. Here, the range of 2790 to 3020 cm$^{-1}$ was scanned with a step size of 15 cm$^{-1}$, covering two $CH_2$ stretching resonances at 2845 cm$^{-1}$ (symmetric stretching, depicted in yellow) as well as the 2920 cm$^{-1}$ resonance (anti-symmetric stretching mode, depicted in magenta), which highlights lipid and protein contributions, respectively. In addition, the contrast mechanism was changed from CARS to SRS, which is technically a more challenging approach, but offers the advantage that it does not contain non-resonant background contributions, which are intrinsic to the CARS signal generation process. Once a stack of images has been acquired, where for each image the pump beam was spectrally shifted by 15 cm$^{-1}$, an SRS spectrum can be generated for each pixel of the image. By fitting preselected Raman spectra to the data set, a false-color hyperspectral SRS image (**Figure 7**) can be generated, where specific colors are assigned to individual Raman resonances. Single hepatocytes with their nuclei (orange arrow) as well as an extended amount of lipid droplets (red arrow) can be identified in the liver tissue. Again, the SHG signal was subsequently acquired to highlight the fibrotic regions within the sample (green), which extends from

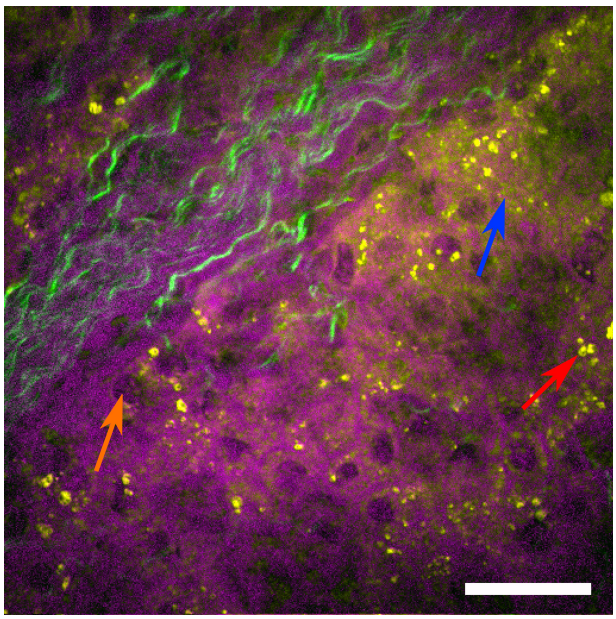

**FIGURE 7 |** Hyperspectral SRS image of human liver biopsy. Hyperspectral SRS image probing the molecular resonances from 2790 to 3020 $cm^{-1}$ (utilizing spectral fitting for every pixel, where amplitude values of the peaks at 2845 $cm^{-1}$ (indicating lipids) are shown in yellow and at 2920 $cm^{-1}$ (indicating proteins) are shown in magenta. Single hepatocytes as indicated by their nuclei (orange arrow), sinusoids as well as lipid droplets (red arrow) can be identified. The green color channel shows the SHG signal indicating fibrotic tissue, which extends from the portal tract into the liver parenchyma. The parenchyma shows microvesicular steatosis (highlighted by a blue arrow). Scale bar is 30 μm.

the portal tract into the liver parenchyma, while the parenchyma exhibits microvesicular steatosis as indicated by the accumulation of a large number of lipid droplets (yellow).

## Super-Resolution Structured Illumination Microscopy of Human Liver Sinusoidal Endothelial Cells

The first description and electron microscopic observation of LSEC fenestrae was given by Wisse (1970). Today, half a century later, advanced optical microscopy techniques have evolved which allow us to resolve these nanoscale features in fresh, live and/or fixed cultures of cells (Schermelleh et al., 2019). Super-resolution structured illumination microscopy (SR-SIM) is a super-resolution microscopy (SRM) technique that is particularly attractive for imaging LSEC (Gustafsson et al., 2008; Schermelleh et al., 2008). In the linear implementation of SR-SIM, rather than illumination the sample with an even, homogeneous illumination, an interference pattern is created within the sample. If the interference pattern has a periodicity close to the smallest spatial feature that can be resolved by the microscope objective lens, then SR-SIM achieves approximately twice the spatial resolution obtained with high-resolution conventional fluorescence microscopy, i.e., approximately 100 nm laterally (Heintzmann and Huser, 2017). Alternatively, by creating a sinusoidal interference pattern with optics using high index of refraction materials, a spatial resolution below 90 nm was demonstrated (Li et al., 2015). In a non-linear implementation, where rather than a slowly varying sinusoidal illumination, a pattern with steep edges is created in the sample and a spatial resolution of less than 50 nm has been achieved (Gustafsson, 2005; Rego et al., 2012; Li et al., 2015). Currently, however, non-linear SR-SIM requires either saturating the fluorescence excitation or the use of photoswitchable fluorophores (Gustafsson, 2005; Rego et al., 2012). Both of these restrictions currently prohibit their use with LSECs: saturating fluorescence requires high laser power, which is detrimental to cell health and the fluorescence photobleaches rapidly. And the genetic modifications required to incorporate photoswitchable fluorescent proteins could, at best, only be done with animal models and are not possible with human LSEC. A last issue is that LSEC fenestrae cannot be labeled directly and, instead the plasma membrane needs to be stained. Linear SR-SIM, on the other hand, does not require specific properties of fluorescent probes and works with most fluorophores, it can easily be extended to multiple colors (Schermelleh et al., 2008) and it is fast - enabling even the imaging of living cells at video rate (Markwirth et al., 2019). The ∼ 100 nm lateral spatial resolution is sufficient to visualize fenestrations in LSEC as was originally shown in fixed rat LSECs (Cogger et al., 2010; Svistounov et al., 2012). Here, we demonstrate this ability by imaging fenestrations in human LSEC (hLSEC). In order to visualize fenestrae, the plasma membrane of fixed hLSECs was stained with an orange-fluorescent membrane stain. The result can be seen in the series of images shown in **Figure 8**. Here, 3 hLSEC out of a series of > 50 cells that were imaged in a single session are shown, which clearly exhibited groups of fenestrae organized in so-called sieve plates. Enlarged versions of such sieve plates are shown next to the full cell SR-SIM images and correspond to the regions of interest outlined by dashed white squares. As can be seen from these images, sieve plates occur primarily in the extended parts of the plasma membrane far from the nucleus, where the distance between the basal and apical membrane is typically the thinnest. Also, in stark contrast to rat LSECs, where sieve plates can occupy up to 60% of the entire cell's surface (Mönkemöller et al., 2015), a significantly smaller fraction of the membrane of hLSEC is covered by sieve plates. We attribute this to defenestration due to the old age of the human patient from which these samples were obtained, as well as potentially underlying health conditions affecting the health of these rather sensitive cells (Couteur et al., 2008).

In order to obtain a quantitative measure of the size distribution of fenestrae in hLSEC, we identified and measured fenestrae in 5 different hLSEC using an automated image processing macro written in Python. Previous distributions of fenestration sizes were obtained by hand and the automation of this process can be difficult because of the significant variations in local brightness due to uneven staining of the cells, as is apparent from the images shown in **Figure 8**. The Python macro utilizes an adaptive thresholding process to identify fenestrae. Specifically, images are first opened, expanded to double the original pixel count, and then dilated with a filter

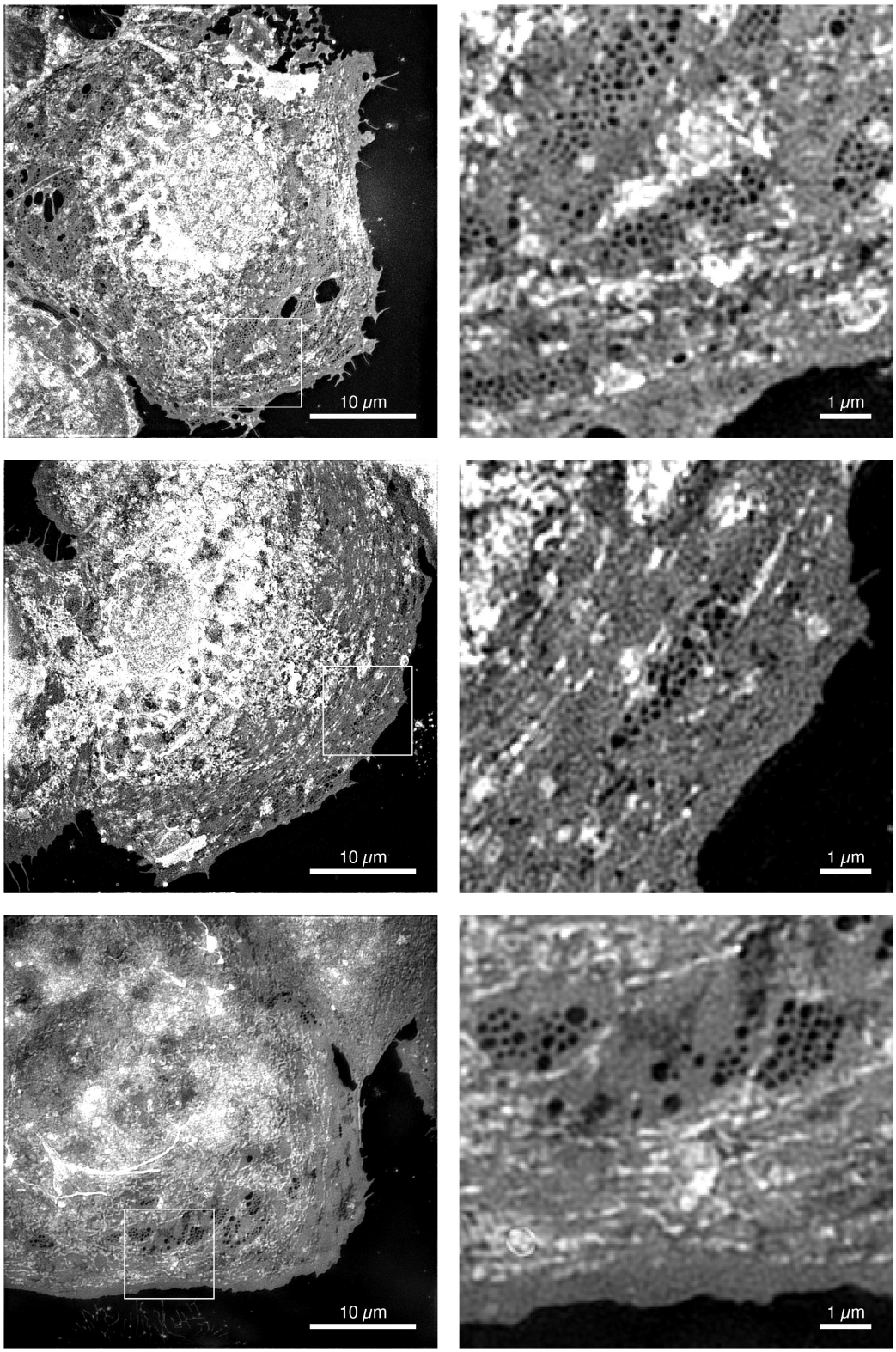


**FIGURE 8 |** Super-resolution structured illumination micrographs of human LSECs. Super-resolution structured illumination microscopy images of 3 different human liver sinusoidal endothelial cells. The cells were stained with the membrane dye CellMask Orange, which allows the visualization of fenestrae as dark holes. The first column shows images with a full field-of-view of 40 μm for each cell. The second column shows magnified views of the white outlined boxes in the images to the left typically displaying one or more sieve plates.

corresponding to the doubled pixel number. This process evens out the brightness distribution. The image is then inverted and the adaptive thresholding process applied. Here, the user can choose the threshold value, which needs to be adjusted from image to image. Subsequently, local maxima are found, and adjacent, potentially overlapping fenestrae are separated by watershedding. Finally, the fenestrae are segmented and measured. Fenestrae with a diameter < 95 nm were excluded, because of the spatial resolution limit of SR-SIM, and fenestrae with a diameter > 320 nm were also excluded, because these are considered to be holes in the membrane rather than fenestrae. The outcome of this process is demonstrated on the example region of interest shown in **Figures 9A,B**. Here, the same region of interest as shown in the lowest row of **Figure 8** was selected and the fenestration finding macro was applied to the region of interest. **Figure 9B** shows fenestrae that were identified by this macro highlighted by yellow circles. The diameter of these circles highlighting fenestrae corresponds to double the measured diameter, which enables the easier identification of the underlying fenestrae by the human eye. The diameters are also written into a text file. We have applied this process to a total of 21 regions of interest selected from the 5 human LSECs where sieve plates were most clearly visible. This resulted in 4471 fenestrae being identified. Their size distribution is shown in the histogram in **Figure 9C**. As can be seen from this histogram, in the human LSEC the distribution of fenestrae diameters is falling off exponentially in the range between 90 – 320 nm and it peaks in the range between 110 – 120 nm. This presents the first measurement of fenestration diameters in hLSEC by SRM under aqueous conditions.

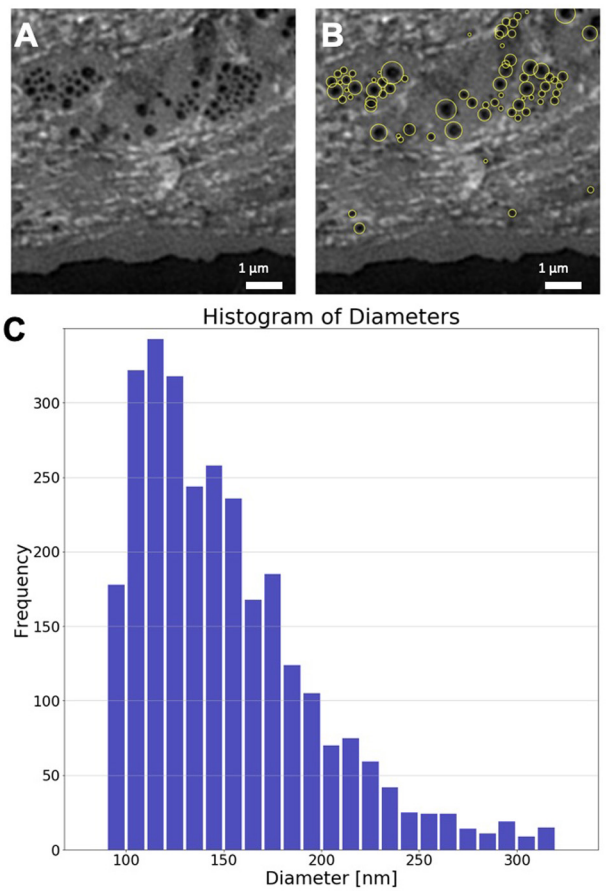


**FIGURE 9 |** Analysis of hLSEC fenestration diameters. **(A)** Region of interest of an SR-SIM image depicting several sieve plates in the plasma membrane of hLSEC. **(B)** The same image as shown in **(A)**, where fenestrae that were automatically identified and sized are highlighted by yellow circles, where the circle diameter corresponds to double the diameter identified for each fenestra. **(C)** Size distribution histogram of 4471 fenestrae identified in 21 regions of interest taken from 5 hLSECs.

## CONCLUSION

In conclusion, we have demonstrated the optical imaging of liver morphology and liver ultrastructure across 7 orders of magnitude. Mesoscopic imaging techniques, such as optical projection tomography and light sheet fluorescence microscopy were used to produce three-dimensional maps of liver tissue. These methods allowed us to image the bile ducts, as well as blood vessels in optically cleared liver tissue with dimensions of a few millimeters by highly specific fluorescence contrast. The spatial resolution of LSFM was sufficient to identify the affiliation of the blood vasculature with the arterial or portal venous tree based on smooth muscle cell orientation. Remarkably, the smallest bile ducts originating at the hepatocyte canaliculi revealed a reticular network which extends a single or few ductuli toward the portal ducts. LSFM even enabled us to image the lumen in the small bile ductules at the edge of the portal field and to identify individual cholangiocytes in the bile ducts. Confocal fluorescence microscopy then seamlessly extended the spatial resolution to the subcellular scale and allowed us to image the inner structure of cholangiocytes and fibers of the portal field extracellular matrix. Label-free confocal microscopy, in particular a combination of coherent Raman scattering (CRS) together with second harmonic generation, allowed us to image a portal vein with erythrocytes attached to the vessel wall as well as nearby fibrotic tissue without fluorescent staining. Hyperspectral CRS imaging was then used to identify single hepatocytes and microvesicular hepatosteatosis based on the accumulation of lipid droplets, as well as fibrotic liver tissue which extended from the portal tract into the liver parenchyma. Lastly, the submicroscopic structure of human liver sinusoidal endothelial cells was imaged by super-resolution structured illumination microscopy. This allowed us to identify hLSEC with fenestrations and to determine the size distribution of fenestrae in hLSEC, which were measured to exhibit the largest fraction of diameters in the range between 110 – 120 nm. Even imaging of the fenestration dynamics of living hLSECs should be possible with linear SR-SIM, because of the availability of live cell plasma membrane stains. Extensions of the methods presented here, such as the combination of light sheet fluorescence microscopy with super-resolution optical microscopy will, in the near future, enable us to combine many of the different methods discussed here within a single

instrument. This combination is expected to allow us to image the ultrastructure of the liver in extended tissue and it will further improve the quantitative imaging of veins, bile ducts, and sinusoids and our detailed understanding of their connections.

## DATA AVAILABILITY STATEMENT

The datasets generated and analyzed for this study can be found on Zenodo.org using the DOI: 10.5281/zenodo.4300689.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee Münster, Germany, 2017-522-f-S. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

CK, SB, CP, ML, CØ, VM, and WH acquired data. All authors helped analyze the data. All authors were involved in writing the manuscript. FK, TH, and JS perceived the project and supervised the work.

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## SUPPLEMENTARY MATERIAL

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REVIEW
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# The wHole Story About Fenestrations in LSEC

*Karolina Szafranska[1†], Larissa D. Kruse[1†], Christopher Florian Holte[1†], Peter McCourt[1*] and Bartlomiej Zapotoczny[1,2]*

[1] Vascular Biology Research Group, Department of Medical Biology, University of Tromsø – The Arctic University of Norway, Tromsø, Norway, [2] Department of Biophysical Microstructures, Institute of Nuclear Physics, Polish Academy of Sciences, Kraków, Poland

The porosity of liver sinusoidal endothelial cells (LSEC) ensures bidirectional passive transport of lipoproteins, drugs and solutes between the liver capillaries and the liver parenchyma. This porosity is realized via fenestrations – transcellular pores with diameters in the range of 50–300 nm – typically grouped together in sieve plates. Aging and several liver disorders severely reduce LSEC porosity, decreasing their filtration properties. Over the years, a variety of drugs, stimulants, and toxins have been investigated in the context of altered diameter or frequency of fenestrations. In fact, any change in the porosity, connected with the change in number and/or size of fenestrations is reflected in the overall liver-vascular system crosstalk. Recently, several commonly used medicines have been proposed to have a beneficial effect on LSEC re-fenestration in aging. These findings may be important for the aging populations of the world. In this review we collate the literature on medicines, recreational drugs, hormones and laboratory tools (including toxins) where the effect LSEC morphology was quantitatively analyzed. Moreover, different experimental models of liver pathology are discussed in the context of fenestrations. The second part of this review covers the cellular mechanisms of action to enable physicians and researchers to predict the effect of newly developed drugs on LSEC porosity. To achieve this, we discuss four existing hypotheses of regulation of fenestrations. Finally, we provide a summary of the cellular mechanisms which are demonstrated to tune the porosity of LSEC.

**Keywords: fenestration, fenestra, nanopores, LSEC, liver sinusoidal endothelial cells, porosity, liver disease, drug response**

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*Leo A. van Grunsven, Vrije Universiteit Brussel, Belgium*

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*Savneet Kaur, The Institute of Liver and Biliary Sciences (ILBS), India*
*Edward N. Harris, University of Nebraska System, United States*

***Correspondence:**
*Peter McCourt*
*peter.mccourt@uit.no*

*[†] These authors have contributed equally to this work and share first authorship*

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## INTRODUCTION

Within the human body, the main blood-organ barrier is made up of a single layer of thin endothelial cells. In the liver, the microcirculation has a unique morphology that facilitates bi-directional exchange of substrates between hepatocytes and blood in the liver sinusoids (Cogger and Le Couteur, 2009; Fraser et al., 2012). Liver sinusoidal endothelial cells (LSEC) are very thin and perforated with transcellular pores (50–300 nm in diameter) that are also termed as fenestrae or fenestrations (**Figure 1**). These structures were first correctly identified as such with transmission electron microscopy (TEM) by Yamagishi (1959) and described in detail by Wisse (1970). Between 2 and 20% of the LSEC surface is covered by fenestrations which are either scattered individually across the surface or clustered into groups called sieve plates. As there are no diaphragms or

underlying basement membrane, fenestrations make LSEC a highly efficient ultrafiltration system. LSEC thus retain blood cells inside the vessel lumen, whereas small molecules, such as drugs, proteins, lipoproteins, and small viruses can pass this endothelial barrier via fenestrations to reach the surrounding hepatocytes, and vice versa (Fraser et al., 1995a). Fenestrations are therefore a vital structure in liver physiology, providing the primary communication conduit between the liver and the rest of the body, via the circulation. LSEC fenestrations, and the effects of various agents upon them, have been studied extensively with electron microscopy. During the last decade new techniques have been developed and became available to investigate fenestrations in cultured LSEC. Super-resolution optical microscopy provided first detailed information about the composition of fenestration (Cogger et al., 2010, 2013; Mönkemöller et al., 2015; Zapotoczny et al., 2019a) while atomic force microscopy (AFM) provided first information about the dynamics of fenestrations *in vitro* (Zapotoczny et al., 2019b, 2020). Such tools will accelerate the development of therapies that can reverse the loss of fenestrations seen in aging and liver fibrosis (DeLeve, 2015; Hunt et al., 2019).

Fenestration loss during aging manifests as changes in the liver microcirculation, in particular within LSEC, which is a likely cause of dyslipidemia (Le Couteur et al., 2002) and insulin resistance in old age (Mohamad et al., 2016). At the morphological level, LSEC in old age have markedly reduced porosity (percent of the cell surface area covered in fenestrations) by about 50% – in other words, old LSEC become "defenestrated" (**Figure 2**). This defenestration results in hampered bi-directional traffic of substrates between the blood and the hepatocytes. Biomolecules such as lipoproteins, or hormones, or drugs (such as statins or insulin) pass less easily through aged LSEC to reach the hepatocytes to be processed and/or exert their effects. For example, older rats showed a significant reduction in the hepatic volume of insulin distribution (Mohamad et al., 2016), showing that fenestrations facilitate insulin transfer to hepatocytes. Another example is the transfer of lipoproteins across LSEC, which was almost totally abolished in livers from old animals, providing a novel mechanism for age-related dyslipidemia and postprandial hyperlipidemia (Hilmer et al., 2005) and is now accepted as a significant factor in age-related hyperlipidemia (Liu et al., 2015). The same applies in the reverse direction across LSEC – biomolecules produced by the hepatocytes need to pass through fenestrations for release into the plasma, and defenestration hinders this process. Age-related LSEC defenestration is also accompanied by altered expression of many vascular proteins including von Willebrand factor, ICAM-1, laminin, caveolin-1 and various collagens (Le Couteur et al., 2008). However, these changes occur without any age-related pathology of hepatocytes or activation of stellate cells (Warren et al., 2011). The sum of all these processes results in a state whereby liver sinusoidal vessels become more like continuous capillaries, but without the other manifestations seen in diseased livers during "capillarization." Age-related defenestration is therefore also termed "pseudocapillarization." Cellular senescence is one hallmark of aging (Robbins et al., 2021), and (Grosse et al., 2020) proposed that LSEC become senescent at 10–12 months of age in mice, as evidenced by the increased expression of the senescence marker p16. Senolytic drugs (which selectively kill senescent cells) have been proposed as a potential therapy to alleviate the effects of senescent cell mediated aging and disease (Robbins et al., 2021). However, $p16^{high}$ LSEC are essential for mouse healthspan, as ablation of these cells results in disruption of the hepatic sinusoid and liver fibrosis (Grosse et al., 2020).

Defenestration of LSEC also occurs during chronic liver disease, liver fibrosis and consequently cirrhosis, which are an increasing worldwide problem, and are becoming a major cause of morbidity and death (Asrani et al., 2019). Currently, there is no therapy that can alleviate fibrosis progression or reverse fibrosis (Higashi et al., 2017). Fibrosis is characterized by excessive extracellular matrix production from activated stellate cells. In addition to LSEC defenestration, during chronic liver disease, a basement membrane develops in the Space of Disse, leading to the process of capillarization, and thereby further reducing the free passage of substrates to and from the hepatocytes (Poisson et al., 2017). Defenestration of LSEC occurs earlier than the formation of fibrous septa in liver diseases such as alcoholic liver injury and non-alcoholic fatty liver disease (Horn et al., 1987) which could indicate that LSEC can play an important role during the early stages of fibrosis. Restoration of differentiation to LSEC led to quiescence of hepatic stellate cells and regression of fibrosis in thioacetamide challenged rats (Xie et al., 2012b) potentially suggesting that therapies that revert LSEC from a diseased/defenestrated state to a normal state may also be of benefit for treatment of liver fibrosis (DeLeve, 2015).

As mentioned above, defenestration of the liver sinusoidal endothelium impairs the hepatic clearance of pharmacological agents (Mitchell et al., 2011). As for lipoproteins and insulin, fenestrations are conduits for pharmaceuticals, from the plasma to the hepatocytes. Reduction in LSEC porosity thus reduces the passage of drugs to the cells where they are processed and metabolized. This can result in elevated and potentially toxic concentrations of drugs in the elderly (and patients with liver disease), when administering drug doses appropriate for healthy young people. In addition, polypharmacy is becoming a major issue in the aging population, with over 42% of people over 65 years of age were reported being administrated five or more different medications per day (Midão et al., 2018). The majority of these medications need to cross the liver sinusoidal endothelium to be detoxified, and it is possible that some of the polypharmacy "cocktails" are detrimental for LSEC porosity. Another serious consequence of reduced porosity is that statins are less able to reach the hepatocytes and inhibit cholesterol production. Increased statin doses are then required to achieve therapeutic effects, sometimes resulting in side effects such as muscle pain and rhabdomyolysis, resulting in medication non-compliance in patients.

Given the vital role of LSEC fenestrations (and the bi-directional flow of substrates through them) in physiology and homeostasis, a better understanding of how these structures are regulated will enable us to design novel therapeutic approaches targeting biological changes of aging and liver diseases.

It needs to be highlighted, however, that many reports in the literature "suffer" from developing experimental methodologies.

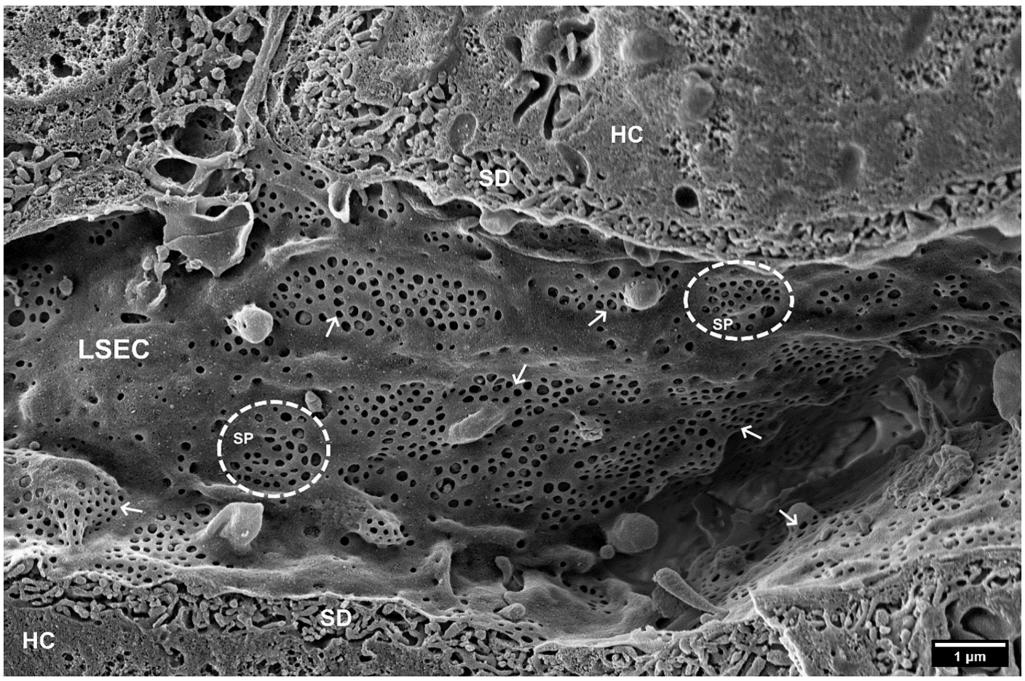


**FIGURE 1 |** SEM image of hepatic sinusoids of a C57BL6 mouse, approximately 4 months old. Liver Sinusoidal Endothelial Cells (LSECs) are covered in multiple fenestrations (arrows) arranged into sieve plates (SP, dotted line circles) distributed over the whole sinusoid. SD, space of Disse; HC, hepatocytes. (Courtesy of Karen K. Sørensen, UiT, Tromsø, Norway).

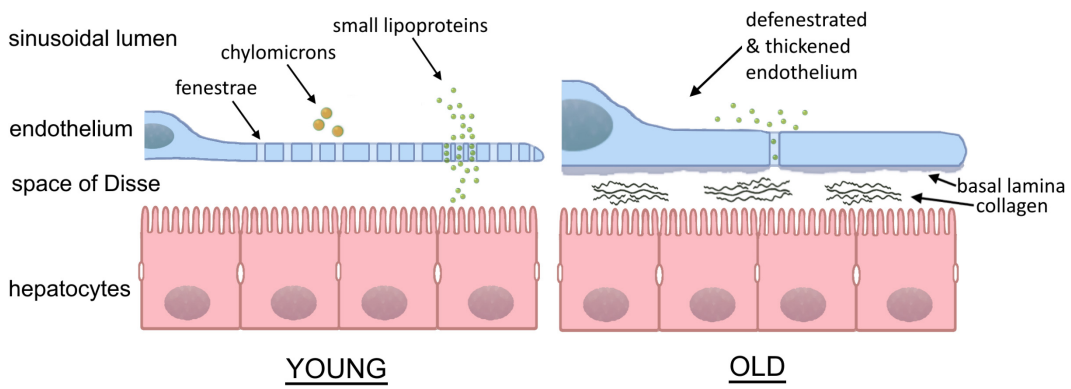


**FIGURE 2 |** Sinusoidal lumen in young and old liver. With age, the fenestrated morphology of the sinusoids is lost in the process of "pseudocapillarization." Additionally, the endothelium thickens and collagen deposits can be found within the space of Disse. The result is the inhibition of transfer between the blood and hepatocytes. (Courtesy of Eike Struck, UiT, Tromsø, Norway and David Le Couteur, ANZAC Research Institute, Sydney, Australia).

Errors during liver perfusion, cell isolation methodologies and sample preparations may lead to altered cell phenotypes. Also, it should be noted that studies from pre-super-resolution era where light microscopy was the only technique used for quantification of fenestrations may be imprecise. As reported, fenestrations in LSEC are in the range of 50–300 nm, gathered in sieve plates of several to tens of pores, with limited number of gaps (DeLeve and Maretti-Mira, 2017). These can be visualized only using non-diffraction limited methods such as electron microscopy, optical nanoscopy, or atomic force microscopy. The distribution of fenestration diameter in this range was presented for both LSEC in tissue (*in vivo*) and for isolated cells (*in vitro*). *In vivo* data are limited to fixed and dried material, while data for isolated LSEC covers fixed and dried, wet-fixed, and live cells. Recently, we summarized that the differences in mean fenestration diameter for fixed and dried, wet-fixed and live LSECs *in vitro* can be up to 30% (Supplementary Table 1 in Zapotoczny et al., 2019b). The differences between *in vivo* and *in vitro* data can be even larger *ibid*., (Wisse et al., 2010). The comparison between the groups in a single report provides information about the alterations as the same microscopy method is applied. The methodological details enabling avoiding errors in imaging and data analysis

were described: SEM (Wisse et al., 2010; Cogger et al., 2015; Szafranska et al., 2021), AFM (Zapotoczny et al., 2017a, 2020; Szafranska et al., 2021), SIM (Kong et al., 2021; Szafranska et al., 2021). Moreover, the comparative measurements using different microscopies were reported in the past showing good correlation between the methods. However, the comparative analysis of newly developed techniques applied recently for LSECs, such as SIM, STED, and AFM, is lacking. Each method has its advantages and limitations. To enable easy tracking of the model (*in vivo/in vitro* and microscopy technique) we provide the relevant information in the presented tables.

The purpose of this review is to: (i) provide a medical and cell biology "tool-kit," for researchers and clinicians to design potential LSEC refenestration strategies and (ii) summarize the existing knowledge around fenestration biology which can help to find new ways to reveal how fenestrations actually work. The first part of this review fucuses on the reported influence of drugs on LSEC fenestration number and porosity, while the second part gives a deeper knowledge about fenestration biology and mechanisms behind structure, formation and maintenance of fenestration. This review does not cover a number of other aspects of LSEC biology, but these can be found in in the following excellent reviews about LSEC in: diseases (Gracia-Sancho et al., 2021; Wang and Peng, 2021), hepatic fibrosis (DeLeve, 2015), mechanotransduction (Shu et al., 2021), inflammation and cancer (Wilkinson et al., 2020; Yang and Zhang, 2021), receptor expression (Pandey et al., 2020), immunological functions (Shetty et al., 2018), aging (Hunt et al., 2019), scavenging (Sørensen et al., 2012), and overall biology of LSECs (Sørensen et al., 2015).

# LSEC AND DRUG INTERACTIONS

## Recreational and Medicinal Drugs, and Their Effects on LSEC Porosity

The human race already uses an extensive array of drugs for medical and recreational purposes. The majority of these compounds are safe, or at least relatively safe for normal human consumption if used appropriately. Reported negative side-effects of these drugs are typically well-documented at the systemic or organ level, but little is known about their direct effects on LSEC fenestration status. Additionally, some drugs with other intended targets may actually have positive side effects on LSEC fenestration, leading to increased LSEC porosity and improving bi-directional exchange of solutes between hepatocytes and plasma. This concept was first tested by Hunt et al. (2019, 2020) who found that a number of drugs for intended use for the treatment of high blood pressure, erectile dysfunction and diabetes improved LSEC porosity in young and old mice. **Table 1** lists the effects of some recreational and medicinal drugs on LSEC fenestrations.

### Recreational Drugs

The effects of recreational drugs on LSEC porosity have not been studied extensively (**Table 1**). The few studies performed showed that the recreational drugs nicotine, ethanol, and cocaine reduce LSEC porosity (Fraser et al., 1988; McCuskey et al., 1993), while the psychedelic drug 2,5-Dimethoxy-4-iodoamphetamine (DOI) increases porosity in LSEC in young and old rodents (Cogger et al., 2014; Hunt et al., 2019). The effects on LSEC porosity of other recreational/non-medicinal drugs such as opioids, amphetamines, cannabis, and xanthines (such as caffeine and theobromine) have, to the best of our knowledge, not been studied. This would be an area of great interest, given the extensive use of all of these among the general population. This is exemplified by opioid use (which is also for medicinal purposes) leading to the current "opioid epidemic" in the US arising from the use of prescription oxycodone. Below is a summary of the reported interactions of ethanol, cocaine, DOI, and nicotine with LSEC.

**Ethanol** Given the wide use and general acceptance of alcohol, and the suggested health benefits from moderate consumption, it was discussed in the LSEC field whether moderate amounts of alcohol could improve LSEC porosity and thereby lipoprotein clearance. Of the studies (*in vitro* and *in vivo*) investigating the effects of ethanol on LSEC, the majority were performed in rats, but mice, baboons and human LSEC were studied as well, with electron and atomic force microscopy methods used as readout. Several studies reported that the fenestration number was reduced, while the average fenestration diameter was increased – this pattern was consistent in all the *in vitro* studies (Mak and Lieber, 1984; Charles et al., 1986; Van Der Smissen et al., 1986; Horn et al., 1987; Tanikawa et al., 1991; McCuskey et al., 1993; Braet et al., 1994, 1995a, 1996c; de Zanger et al., 1997) and with reduced porosity reported in one study (Takashimizu et al., 1999). Takashimizu et al. (1999) described reduction in fenestration diameter in rat during *in vivo* continuous administration of ethanol into the portal vein, and pre-treatment with **BQ123** [an endothelin (ET) receptor antagonist, see **Table 2**] reduced the effect of ethanol. One *in vivo* study reported no changes in in the liver sinusoids in mice after 9 weeks of ethanol feeding (McCuskey et al., 1993) but ethanol in combination with cocaine caused the sinusoids to become thickened and defenestrated. In other *in vivo* chronic ethanol challenge studies (ethanol given to rats in food, or human studies where biopsies were used), one rat study yielded results consistent with the *in vitro* findings (reduced fenestration number, increased diameter, reduced porosity) (Tanikawa et al., 1991) while the other study reported reduced fenestration diameter and number – this was the only study to find that the diameter became smaller after ethanol challenge (Takashimizu et al., 1999). In the human biopsy study, similar results were obtained - chronic alcohol consumption (defined as > 60 g alcohol intake every day for more than 3 years) resulted in fewer fenestrations, diameters of between 50–300 nm and a "visible difference" for porosity between the two groups. A study in baboons showed that the duration of alcohol consumption does not seem to have any impact on fenestrations (diameter in second group (4–24 months alcohol consumption vs. 61–112 months) was larger than control but smaller than first group) (Mak and Lieber, 1984). In summary, ethanol at any dose does not appear to improve LSEC porosity but rather has the opposite effect.

**TABLE 1 |** Influence of medicinal drugs on LSEC fenestrations.

| | Fenestration diameter | Porosity | Fenestration frequency | References | Methods |
|---|---|---|---|---|---|
| **Recreational drugs** | | | | | |
| Ethanol | +/- | - | - | Van Der Smissen et al., 1986; Braet et al., 1995a | SEM, TEM, *in vitro* |
| | | | | Mak and Lieber, 1984; Charles et al., 1986; de Zanger et al., 1997 | SEM, *in vivo* |
| | | | | Tanikawa et al., 1991; McCuskey et al., 1993 | TEM, *in vivo* |
| | | | | Horn et al., 1987; Takashimizu et al., 1999 | SEM, *in vivo* |
| | | | | Braet et al., 1996c | SEM, AFM, *in vitro* |
| | | | | Braet et al., 1994 | SEM, *in vitro* |
| Ethanol +cocaine | nd | - | - | McCuskey et al., 1993 | TEM, *in vivo* |
| Cocaine | nd | nd | nd | McCuskey et al., 1993 | TEM, *in vivo* |
| 2,5-Dimethoxy-4-iodoamphetamine (DOI) | + | +/- | +/- | Furrer et al., 2011; Cogger et al., 2014 | SEM, *in vivo* |
| | | | | Hunt et al., 2019 | SEM *in vitro* |
| Nicotine | - | - - | - | Fraser et al., 1988 | SEM, *in vivo* |
| **Prescription drugs** | | | | | |
| Acetaminophen/paracetamol +ethanol | G | nd | nd | McCuskey et al., 2004 | SEM, TEM, *in vivo*, *in vitro* |
| Acetaminophen/paracetamol | G | - | - | Ito et al., 2006b | SEM, *in vivo* |
| | | | | Walker et al., 1983 | SEM, TEM, *in vivo* |
| | | | | McCuskey et al., 2004; McCuskey, 2006 | SEM, TEM, *in vivo*, *in vitro* |
| Amlodipine | - | + | + | Hunt et al., 2019 | SEM, *in vitro* |
| Bosentan | 0 | + | + | Hunt et al., 2019 | SEM, *in vitro* |
| Colchicine | nd | nd | 0 | Braet et al., 1996b | TEM, *in vitro* |
| Disulfiram | - | nd | + | Bernier et al., 2020 | SEM, *in vivo* |
| Metformin | 0 | + | + | Hunt et al., 2020 | SEM, *in vitro*, *in vivo* |
| | | | | Alfaras et al., 2017 | SEM, *in vivo* |
| Nicotinamide mononucleotide (NMN) | 0 | + | + | Hunt et al., 2019 | SEM, *in vitro* |
| | | | | Mao et al., 2019 | dSTORM, *in vitro* |
| Cholesterol | 0 | 0 | 0 | Fraser et al., 1988, 1989 | SEM, *in vivo* |
| Cholesterol +nicotine | - | - | - | Fraser et al., 1988 | SEM, *in vivo* |
| Pantethine + cholesterol | + | + | + | Fraser et al., 1989 | SEM, *in vivo* |
| Prostaglandin E1 | + | | | Oda et al., 1997 | SEM, TEM, *in vitro* |
| Sildenafil | 0/+ | ++ | + | Hunt et al., 2019 | SEM, *in vitro* |
| | | | | Mao et al., 2019 | dSTORM, *in vitro* |
| Simvastatin | + | + | + | Hide et al., 2020 | SEM, TEM, *in vivo*, SEM, *in vitro* |
| | | | | Venkatraman and Tucker-Kellogg, 2013; Hunt et al., 2019 | SEM, *in vitro* |
| Taxol | nd | nd | 0 | Braet et al., 1996b | TEM, *in vitro* |
| TNF-related apoptosis-inducing ligand (TRAIL) | +/0 | +/0 | +/0 | Hunt et al., 2019 | SEM, *in vitro* |

*"0," no change; G, gaps; increase: "+," <50%; "++," 50–100%; "+++," >100%; decrease: "-," <50%; "- -," >50%; "- - -," defenestration; "nd," no data.*

**Cocaine** is a widely used recreational drug with vasoconstricting properties (Kim and Park, 2019), often consumed in combination with alcohol. In a study from McCuskey et al. (1993), mice challenged with cocaine alone developed basement membrane deposition in the space of Disse, some hepatocellular necrosis and slightly reduced centrilobular sinusoid blood flow after 5 weeks, worsening up to 9 weeks of challenge. In combination with ethanol these changes were significantly exacerbated, in addition the sinusoidal endothelium was thickened and defenestrated. Interestingly rats were more resistant to these challenges, only developing some of these changes at the end of the 15-week treatment regime. The

**TABLE 2 |** Influence of hormones and other agents acting on LSEC fenestrations.

| | Fenestration diameter | Porosity | Fenestration frequency | References | Methods |
|---|---|---|---|---|---|
| **Vasoactive stimuli** | | | | | |
| **Vasodilators** | | | | | |
| Acetylcholine | + | nd | nd | Tsukada et al., 1986; Oda et al., 1990 | SEM, *in vivo*, *in vitro* |
| Bethanechol | + | nd | nd | Oda et al., 1990 | SEM, *in vivo* |
| Isoproterenol | + | nd | nd | Oda et al., 1990 | SEM, *in vivo*, *in vitro* |
| Vasoactive intestinal peptide (VIP) | + | nd | nd | Oda et al., 1990 | SEM, *in vivo* |
| BQ-123 | ++ | nd | - | Watanabe et al., 2007 | SEM, TEM, *in vivo* |
| **Vasoconstrictors** | | | | | |
| Endothelin (ET) | - | - | nd | Oda et al., 1997; Kamegaya et al., 2002 | SEM, *in vitro* |
| Neuropeptide Y | - | nd | nd | Oda et al., 1990 | SEM, *in vivo* |
| Norepinephrine/ noradrenaline | - | nd | nd | Tsukada et al., 1986; Oda et al., 1990 | SEM, *in vivo*, *in vitro* |
| | | | | Wisse et al., 1980 | TEM, SEM, *in vivo* |
| Serotonin | - | nd | nd | Wisse et al., 1980; Braet et al., 1995a | SEM, TEM, *in vivo* |
| | | | | Tanikawa et al., 1991 | TEM, *in vivo* |
| | | | | Braet et al., 1996c | SEM, AFM, *in vitro* |
| | | | | Kalle et al., 1997 | AFM, *in vitro* |
| Pilocarpin | - | nd | nd | Wisse et al., 1980 | TEM, SEM, *in vivo* |
| Adrenaline/ epinephrine | - | nd | nd | Wisse et al., 1980 | TEM, SEM, *in vivo* |
| **Signaling/Maintenance** | | | | | |
| Vascular endohelial growth factor (VEGF) | + | +++ | ++ | Funyu et al., 2001; Yokomori et al., 2003 | SEM, *in vitro* |
| | | | | Carpenter et al., 2005 | SEM, TEM, *in vivo* |
| | | | | Xie et al., 2012b | SEM, *in vivo*, *in vitro* |
| Bone morphogenetic protein (BMP) | Strain specific | Strain specific | Strain specific | Desroches-Castan et al., 2019a,b | (a) SEM, *in vivo*, *in vitro* (b) SEM, *in vitro* |
| Platelet derived growth factor (PDGF-B) signaling | nd | - | nd | Raines et al., 2011 | TEM, *in vivo* |
| Liver X receptor (LXR) | NA | NA | NA | Xing et al., 2016 | SEM, TEM, *in vivo* |
| Hedgehog (Hh) signaling | nd | - | nd | Xie et al., 2012a | SEM, *in vitro* |
| Plasmalemma vesicle associated protein (PLVAP) | +/- | +/- | +/- | Herrnberger et al., 2014 | SEM, TEM, *in vivo* |
| | | | | Auvinen et al., 2019 | SEM, *in vivo* |

*"0," no change; G, gaps; "nd," no data; "NA," not applicable. increase: "+," <50%; "++," 50–100%; "+++," >100%; decrease: "-," <50%; "--," >50%; "---," defenestration.*

mechanism(s) by which cocaine and cocaine/ethanol challenge elicit these changes remains to be elucidated, but in any case the combined abuse of these drugs raises particular concerns with regards to liver function.

**Nicotine** is the primary stimulant found in tobacco products and is also a known vasoconstrictor (Benowitz and Burbank, 2016). Rats fed nicotine (dose equivalent to 50–100 cigarettes per day in humans for 6 weeks) had LSEC porosity 40% of that of controls, primarily as a function of reduced average fenestration diameter and not of reduced fenestration number. The nicotine treated animals also had near 50% higher serum cholesterol than controls, probably as a consequence of reduced LSEC porosity and thereby filtration of low-density lipoprotein (LDL) out from the plasma of these animals (Fraser et al., 1988). Nicotine and cholesterol fed animals had similar porosity and diameter to nicotine-fed only animals. Together with results from cholesterol-only fed animals (no visible changes), it suggests that nicotine (but not cholesterol) has an effect on fenestrations (Fraser et al., 1988). Other studies have shown that oral nicotine induces an atherogenic lipoprotein profile (Cluette-Brown et al., 1986) (including increased plasma LDL) and impairs plasma LDL clearance (Hojnacki et al., 1986). The mechanism of action

of nicotine in the LSEC context remains to be elucidated but given the continued consumption of nicotine by humans in various forms (e.g., tobacco products, e-cigarettes, and nicotine supplements) this field warrants further study.

**2,5-Dimethoxy-4-iodoamphetamine** (DOI) is a substituted amphetamine but is not a stimulant. It is a potent 5-$HT_{2A}$ serotonin receptor agonist and is used recreationally as a hallucinogenic drug (Lapoint et al., 2013). DOI induces cutaneous vascular constriction in rabbits and rats, and this is the suggested cause of hyperthermia resulting from serotonin receptor stimulation (Blessing and Seaman, 2003). DOI has reported beneficial effects on survival, liver regeneration and LSEC morphology after partial hepatectomy (Tian et al., 2011). Furrer et al. (2011) showed that *in vivo* DOI challenge increased porosity in old but not young LSEC, and pre-treatment of old mice with DOI prior to partial hepatectomy resulted in LSEC with improved porosity (Furrer et al., 2011). However, the finding that DOI improved porosity in aged LSEC is at odds with the *in vivo* study of Cogger et al. (2014) who found that DOI improved LSEC porosity in young but not old animals. Both studies used SEM of tissue blocks to quantify fenestrations. Further complicating the DOI story, SEM *in vitro* studies by Hunt et al. (2019) on cultured LSEC from young and old mice revealed that DOI challenge increased porosity in old but not young LSEC, and this increase was most likely a function of increase in both fenestration diameter and frequency. LSEC respond to ligands for the 5-HT2 receptor, as they were reported to being inhibited by ketanserin (a selective 5-HT2 receptor antagonist) (Gatmaitan et al., 1996). The role of 5-HT2A and 2B receptors was proposed as being involved in liver regeneration after liver partial hepatectomy (Lesurtel et al., 2006). Similarly, the presence of the 5HT2 receptor was later highlighted (Braet and Wisse, 2002; Braet, 2004). However, newly reported data showed that known 5-HT receptor mRNAs were absent or at very low levels in mouse, rat and human LSEC (Bhandari et al., 2020). It would thus be of interest to resolve the question of DOI mediated effects, the downstream mechanisms, and whether there is/are age-related responses to DOI.

## Medicinal Drugs

Pharmaceutical treatment and prevention of diseases is constantly evolving, with an increasing number of novel medicines entering the market every year. It was reported that the EU retail pharmaceutical bill was around EUR 190 billion in 2018 (OECD/European Union, 2020). Hepatic clearance and metabolism are the basic routes of removing drugs from the system. With decreased porosity prolonged circulation of drugs increases their side effects. Nitric oxide (NO)-based drug therapy was shown to have beneficial effects on the liver (Maslak et al., 2015) and detailed studies on isolated cells confirm the positive role of NO on fenestrated morphology in LSEC (Xie et al., 2012b). Medicinal drugs with other intended targets may also affect LSEC. A recent comparative study revealed the different drug effects on fenestrations in LSEC in an age-related manner (Hunt et al., 2019). Here we summarize the effects of various medicines where fenestration number and size were reported.

**Amlodipine** is a calcium channel blocker used to treat hypertension by dilating blood vessels to reduce blood pressure. Amlodipine is also reported to increase endothelial NO (Xu et al., 2002; Mason et al., 2014). Hunt et al. (2019) reported that amlodipine increased the porosity in cultured LSEC from both young and old animals and proposed that this increase was more likely mediated by NO production than by calcium transport blockage. This safe and commonly used blood pressure medicine may thus also represent a pharmacological means to counteract age-related defenestration.

**Bosentan** is a competitive antagonist of endothelin -A and -B receptors, and is used to treat moderate pulmonary hypertension, exerting its vasodilative effect via ET-A receptors (Bacon et al., 1996). Endothelin-1 (ET-1) constricts fenestrations pronouncedly and reduces porosity (Kamegaya et al., 2002), and an ET-B receptor antagonist (BQ788) blocked this effect while an ET-A receptor antagonist (BQ485) partially blocked the ET-1 effect (Kamegaya et al., 2002). The ET-A receptor antagonist **BQ123** increased fenestration diameters, but caused major gaps in sinusoidal cells and fusions of fenestrations within sieve plates (Watanabe et al., 2007). Hunt et al. (2019) demonstrated that lower doses of bosentan increased the porosity of LSEC from old mice, while LSEC from younger mice were non-responsive. Bosentan treatment of LSEC did not elicit an increase in NO production in this study.

**Colchicine** is used as a therapy for gout and familial Mediterranean fever. It decreases inflammation but its pharmacotherapeutic mechanism of action is not fully understood – its main mechanism of action is tubulin disruption (Leung et al., 2015). Treatment of cultured rat LSEC with 200 μM colchicine did not affect porosity while causing significant loss of microtubules. Interestingly, the microtubules surrounding sieve plates were still present (Braet et al., 1996b). Together with the effect of taxol, which completely disrupts microtubules and prevents cytochalasin-mediated induction of fenestrations, this would suggest that tubulin architecture may have a crucial role in LSEC porosity. **Taxol** (generic name paclitaxel) is a microtubule-stabilizing drug used for the treatment of ovarian, breast, and lung cancer, as well as Kaposi's sarcoma (Weaver, 2014). Braet et al. (1996b) challenged cultured rat LSEC with 10 μM taxol and saw no change in porosity but reported an overabundance of microtubules throughout the cytoplasm, and alongside sieve plates. Moreover, treatment with 10 μM taxol not only did not show a significant change in fenestration number but pretreatment with taxol and two hours later with cytochalasin B, inhibits the effect of the latter, i.e., the increase in fenestration number is reduced in comparison to treatment with cytochalasin B only.

**Disulfiram** (commercial name Antabuse) is a FDA approved treatment for chronic alcohol addiction. It is an inhibitor of acetaldehyde dehydrogenase and causes the feeling of a hangover immediately upon alcohol consumption (Suh et al., 2006). It is an inhibitor of the transcription factor NF-KB (Schreck et al., 1992) which contributes to its anti-inflammatory properties. In the experimental setting, the consumption of disulfiram was found to normalize body weight in mice. It was also found to increase the frequency of LSEC fenestrations *in vivo*, while decreasing

their average diameter, resulting in no net increase in porosity in mice and rats (Bernier et al., 2020). The mechanism(s) by which disulfiram increases fenestration number remain to be elucidated.

**Metformin** is a first line treatment for type II diabetes for serum glucose reduction (Maruthur et al., 2016). The mechanism by which this drug exerts this effect remains to be elucidated, but its primary target appears to be hepatocyte mitochondria via inhibition of complex I of the respiratory chain. Inhibition of gluconeogenesis (Owen et al., 2000) results in the activation of the energy sensor AMP-activated protein kinase (AMPK) leading to increased beta-oxidation of fatty acids. Alfaras et al. (2017) tested 1% metformin administered every-other-week or 2-weeks-every-month to mice – these strategies being chosen to avoid metformin induced nephrotoxicity. They found numerous health benefits, particularly with the every-other-week regime, and that the every-other-week approach also increased porosity in LSEC in 2-year-old mice. Metformin (50 μM) increased LSEC porosity *in vitro* in both young and old mice by 25 and 50%, respectively (Hunt et al., 2020). This increase was due to increases in fenestration frequency (20 and 50%, respectively) since the fenestration diameter remained unchanged. *In vivo* studies in mice treated with 0.1% metformin in their diet increased LSEC porosity/fenestration frequency in young and old mice and reduced the age-related loss of porosity in older mice by 50% (Hunt et al., 2020). The mechanism of metformin action in LSEC, with regards to fenestration status, remains to be established.

**Nicotinamide mononucleotide** (NMN) is a key nicotinamide adenine dinucleotide (NAD+) intermediate. Long-term administration of NMN is reported to mitigate age-related physiological decline in mice (Mills et al., 2016), while short term *in vitro* treatment reverses endothelial dysfunction (Mateuszuk et al., 2020). NMN increased LSEC porosity in young and old mice, via increased fenestration frequency, while the average fenestration diameter was essentially unchanged (Hunt et al., 2019). NMN challenge had no apparent effects on NOS or cGMP levels in LSEC. Analysis of NMN challenged LSEC using direct stochastical optical reconstruction microscopy (dSTORM) revealed that the F-actin within LSEC was more condensed and that the actin rings delineating fenestrations became more pronounced (Mao et al., 2019). The mode of NMN action in LSEC remains to be elucidated – NAD + associates with sirtuins which play a critical role in multiple cellular functions (Imai and Yoshino, 2013) so the study of the role of sirtuins in fenestration biology is therefore warranted.

**Pantethine** is a derivative of vitamin B5 and has been suggested as a therapy for reducing LDL levels (Rumberger et al., 2011). Fraser et al. (1989) studied the effect of pantethine in cholesterol fed rabbits. The pantethine plus cholesterol fed animals had higher LSEC porosity, fenestration diameter and frequency and lower total cholesterol than the animals fed cholesterol alone. **Cholesterol** feeding had no effect on LSEC porosity. The same result had been found in another study (Fraser et al., 1988). Unfortunately, there was no group fed only pantethine, so it would be interesting to establish if pantethine alone increases LSEC porosity and if this can explain (in part) the reported pantethine-mediated reduction of plasma LDL seen in other studies (Fraser et al., 1989; Rumberger et al., 2011).

**Paracetamol** (also known as acetaminophen or commercially as APAP, Panadol) is one of the most widely used analgesic medicines. Acute overdoses of paracetamol can cause lethal liver damage, due to the toxic metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (Hodgman and Garrard, 2012). The consensus is that, *in vivo*, paracetamol reduces rodent LSEC porosity both via reduction of fenestration diameter and frequency at "clinical" doses (Walker et al., 1983; McCuskey et al., 2004; McCuskey, 2006; Ito et al., 2006b). The *in vitro* effect of paracetamol on LSEC was reported to be dependent on NAPQI induced depletion of glutathione levels. In C3H mice, acetaminophen is directly toxic to LSEC via P450 activation, while in Swiss Webster mice the toxic effect on LSEC was indirectly driven by hepatocytes (DeLeve et al., 1997). APAP-induced LSEC injury precedes hepatocellular injury, supporting the hypothesis that LSECs are an early and direct target for APAP toxicity. These findings also suggest that reduced sinusoidal perfusion and increased Kupffer cell activity contribute to the development of APAP-induced liver injury (Ito et al., 2003). Although it was presented that large gaps are formed and the porosity is reduced in LSEC *in vivo*, the effects of paracetamol challenge on LSEC porosity *in vitro* have not been reported.

**Prostaglandin E1** (synthetic form: alprostadil) is a naturally occurring eicosanoid used as vasodilator for several different medical purposes (Kirtland, 1988). Applications include erectile dysfunction (ED) treatment in men who do not respond to PDE5 inhibitors (Hanchanale and Eardley, 2014) and the opening of ductus arteriosus in neonates requiring heart surgery (Singh and Mikrou, 2018). Prostaglandin E1 exerts its effect via the production of nitric oxide which stimulates soluble guanylyl cyclase to increase production of cyclic GMP (cGMP) and/or by the direct binding of prostaglandin to prostaglandin receptors, activating adenylyl cyclase to convert ATP to cyclic AMP (cAMP). The end result is the same in either pathway - decreased intracellular $Ca^{2+}$ (Namkoong et al., 2005). Oda et al. (1997) showed that prostaglandin E1 significantly increased LSEC fenestration diameter in rat LSEC and also caused partial fusion of some fenestrations within sieve plates. They also reported increased $Ca^{2+}$-ATPase on fenestral plasma membrane after prostaglandin E1 challenge and postulated that cytoplasmic $Ca^{2+}$ efflux caused relaxation (and thereby dilation) of LSEC fenestrations.

**Sildenafil** (also known as Viagra) is a vasoactive agent used for the treatment of ED. It is a potent and selective inhibitor of cGMP-specific phosphodiesterase (PDE) type 5, due to its structural similarity to cGMP (Bender and Beavo, 2006). Sildenafil increases cGMP levels by inactivating PDEs that metabolize cGMP to GMP as well as by blocking ABCC5 transport protein responsible for active efflux of cGMP from the cell (Aronsen et al., 2014). cGMP is an intracellular mediator of the NO pathway that can lead to relaxion of the vascular smooth muscle (vasodilation) and thereby increase blood flow (Denninger and Marletta, 1999). Hunt et al. (2019) challenged LSEC from young (3–4 months) and old (18–25 months) mice with sildenafil and found that porosity and fenestration frequency (but not diameter) increased in LSEC from young and old mice. Sildenafil also increased cGMP levels, NO synthesis and levels of

phosphorylated nitric oxide synthase (pNOS). Mao et al. (2019) also challenged LSEC (from young mice) and found that the actin rings (which delineate fenestrations) and actin stress fibers became more pronounced. In contrast to Hunt et al. (2019) and Mao et al. (2019) found that sildenafil increased fenestration diameter on average by 30%. This inconsistency might be due to the methods used – the first study used SEM to score LSEC morphology after dehydration, while the second study used dSTORM on "wet" LSEC samples. Sildenafil (and other PDE and ABC transporters inhibitors) may be an interesting therapeutic option to increase LSEC porosity in the elderly.

**Simvastatin** is a cholesterol lowering agent. Its cholesterol reducing action is via inhibition of 3-hydroxy-3-methylglutaryl (HMG) coenzyme A reductase, the rate limiting enzyme in cholesterol synthesis. Simvastatin also upregulates NO levels suggesting vascular protective effects beyond cholesterol reduction (de Sotomayor et al., 2005; Rikitake and Liao, 2005). Hide et al. (2020) reported that simvastatin was somewhat protective against warm ischemia reperfusion induced LSEC defenestration in (male Wistar) rats, so simvastatin may be able to provide a protective role in maintenance of porosity. Venkatraman and Tucker-Kellogg (2013) showed that simvastatin can antagonize Rho/ROCK (Rho-associated protein kinase) signaling, protecting from the defenestration resulting from activation of this pathway. Moreover, simvastatin treatment led to increase on both porosity and fenestration frequency in (male Wistar) rats. Interestingly these results in rats were not replicated in mice. Findings of Hunt et al. (2019) in (male C57/BL6) mice showed no significant changes in porosity or fenestration frequency in young or old mice, and only a 20% increase in mean diameter in the aged group. These findings may suggest species dependent difference in the simvastatin mechanism of action.

**TRAIL** [tumor necrosis factor (TNF)-related apoptosis-inducing ligand] is a protein ligand reported to induce cell death in transformed cells by binding to "death receptors" (Wiley et al., 1995). It is also reported to induce NO production *via* eNOS (Bartolo et al., 2015). Hunt et al. (2019) reported that LSEC challenged with lower doses of TRAIL increased LSEC porosity and fenestration frequency in young but not old mice. The lack of TRAIL response of old mice LSEC could be explained by reduced expression of TRAIL receptors in older mouse LSEC, but the level of TRAIL receptor expression in young vs. old mice remains to be determined.

## Hormones and Other Agents Acting on LSEC

### LSEC and Vasoactive Agents

Vasoactive signaling molecules commonly act through a receptor induced relaxation in the smooth muscle surrounding the vasculature (Webb, 2003). Signaling is mostly mediated by the NO/cGMP pathway and via intracellular calcium concentrations (Chen et al., 2008). Crucially, whether a stimuli directs toward constriction or relaxation will depend on the tissue specific expression of certain receptors and the presence or absence of inhibition of parallel pathways.

Hepatic sinusoids lack smooth muscle cells but can dilate and contract responding to various vasoactive agents. Moreover, according to the two main studies addressing this issue (Oda et al., 1990; Gatmaitan et al., 1996), LSEC porosity and fenestration diameter seem to correlate with vasodilation or vasoconstriction (**Table 2**). These results suggest that vasodilators and vasoconstrictors have a direct effect upon the fenestrations of LSEC. The lack of super resolution techniques for living cells was one of the main drawbacks at the time of these studies of vasoactive agents' effects on LSEC. It will be therefore beneficial for the field investigate the role of vasoconstriction and dilation in fenestration regulation using live cell imaging techniques, such as AFM, SIM or stimulated emission depletion microscopy (STED).

#### *Vasodilators*

**Acetylcholine** is a vasodilator acting through the cholinergic/muscarinic receptor (Sakai, 1980). In LSEC acetylcholine dilates sinusoids increasing blood flow rate and increasing fenestration diameter (Oda et al., 1990), when administered intravenously. On the other hand, **cholinergic receptor agonists** were also noted to cause narrowing of the sinusoids: **bethanechol, carbachol,** and **pilocarpine** applied topically to the liver caused constriction of the liver microvasculature, but fenestrations were not quantified (Reilly et al., 1982; McCuskey and Reilly, 1993). To further complicate these findings, intravascular admission of pilocarpine decreased while bethanechol increased the fenestration diameter. These differences in the effects can be explained by the expression of certain receptors responding to the same stimuli but having contradictory effects, however, further studies are needed. **Bethanechol** is already used as a therapy for postoperative and postpartum non-obstructive urinary retention, it would therefore be of interest to further study its effects on LSEC porosity (Oda et al., 1990). **Vasoactive intestinal peptide (VIP)** is a class II G-protein coupled receptor ligand (Umetsu et al., 2011). It has multiple physiological effects including vasodilation and increased gut motility during digestion (Iwasaki et al., 2019). VIP was shown to dilate the sinusoids and fenestra, increasing blood flow through the sinusoids which would enhance the uptake of circulating nutrients after a meal (Oda et al., 1990). **Isoprenaline** (also known as isoproterenol) is another vasodilating agent acting as a β-adrenergic receptor agonist. This G-protein is essential for cardiac function (reviewed in Wachter and Gilbert, 2012) and is used to treat bradycardia and (rarely) asthma. The effect on LSEC follows that of other of vasodilating agents increasing in both sinusoidal blood flow and fenestration diameter (Oda et al., 1990).

#### *Vasoconstrictors*

**Serotonin** (also known as 5-HT) is a monoamine neurotransmitter with numerous physiological functions (Berger et al., 2009). Depending on the particular receptors expressed in each vessel wall and surrounding smooth muscle tissue, serotonin can cause vasoconstriction or vasodilation in different vascular beds (Kaumann and Levy, 2006). In the liver, serotonin constricts sinusoids and reduces fenestration size (Wisse et al., 1980; Oda et al., 1990). Gatmaitan et al. (1996)

showed that the effect is mediated by decreasing cAMP and increasing intracellular calcium levels in a matter of seconds. **Endothelin (ET)** is a vasoconstricting peptide that is produced in the endothelium and plays an important role in vascular homeostasis (Kawanabe and Nauli, 2011). In LSEC, it decreases both the number and the size of fenestrations (Kamegaya et al., 2002; Yokomori et al., 2006) and it reduces the blood-flow through the sinusoids (Zhang et al., 1994). Many ET receptor antagonists are used as an efficient treatment for hypertension. ET-A receptor antagonist **(BQ-123**) treatment (but not ET-B receptor antagonists) abolished ET induced defenestration and contraction of fenestrations (Yokomori et al., 2006). Blocking ET-1 activity *in vivo* by BQ-123 led to gap formation shown by SEM and TEM (Watanabe et al., 2007). The α-adrenergic receptor family mediates vasoconstriction and is coupled to guanine nucleotide regulatory proteins (G-proteins) (reviewed in Ruffolo and Hieble, 1994). **α-adrenergic receptor agonists** were found to have different effects on LSEC, **epinephrine** (adrenaline) decreased sinusoidal blood flow and contracted sinusoids and LSEC fenestrations (Oda et al., 1990), while in another study sinusoids were found slightly enlarged, and fenestrations unchanged (Wisse et al., 1980). **Norepinephrine** (noradrenaline) was found to contract sinusoids and fenestrations in both studies (Wisse et al., 1980; Oda et al., 1990). **Neuropeptide Y (NPY)**, another vasoconstrictor generally coupled to G-protein signaling, is involved in various physiological and homeostatic processes (White, 1993) but also inhibits gastrointestinal motility (Holzer et al., 2012). In LSEC, NPY constricts both sinusoid and fenestrations (Oda et al., 1990).

### Signaling and Fenestration Maintenance

One of the most challenging aspects of studying LSEC is the dedifferentiation *in vitro* after cell extraction. LSEC lose their characteristic porous morphology after just few days in culture, significantly restricting time for experiments. There have been many attempts to slow down, stop or reverse that process (Bravo et al., 2019; Di Martino et al., 2019) but the main mechanism(s) behind the loss of fenestrations remain unknown.

**Vascular Endothelial Growth Factor (VEGF)** is a hormone that stimulates acetogenesis and angiogenesis (Apte et al., 2019). In LSEC, VEGF has been shown to increase LSEC porosity *in vitro* (Funyu et al., 2001; Yokomori et al., 2003) as well as to prolong the fenestrated phenotype of cultured LSEC *in vitro* (Xie et al., 2012b). Downregulation of VEGF signaling has been associated with LSEC defenestration, capillarization of sinusoids, and abnormal liver physiology (Carpenter et al., 2005; DeLeve, 2015). DeLeve (2015) showed that VEGF promotes fenestration formation/maintenance *via* NO-dependent and NO-independent pathways. Moreover, VEGF can induce fenestration like structures in other microvasculature, e.g., rat cremaster capillary (Roberts and Palade, 1995).

**Bone Morphogenetic Protein 9** (BMP9, also known as GDF2) is a circulating endothelial quiescence factor (David et al., 2008). In LSEC it has been indicated as necessary for fenestration maintenance and treating cells with BMP9 prolonged fenestrated phenotype in cultured LSEC (Desroches-Castan et al., 2019a). BMP9 knockouts in 129/Ola mice showed very low fenestration frequency compared to WT, without changes to diameters (Desroches-Castan et al., 2019a). However, a follow up study using C57/Black mice did not confirm these results (Desroches-Castan et al., 2019b).

**Platelet derived growth factor B (PDGF)** is a member of the PDGF family of major mitogens for many cell types (Fredriksson et al., 2004). Hepatic vascular permeability was highly increased in PDGF-B retention deficient mice, with a three-fold increase in FITC-dextran absorption and a more fenestrated phenotype (Raines et al., 2011). PDGF-B signaling is involved in pericyte recruitment and function, and stellate cell activation (Raines et al., 2011).

**Liver X receptor (LXR)** is a nuclear receptor expressed in a number of tissues, but with highest expression in the liver (Willy et al., 1995). Oxysterols are natural ligands of LXR and LXR deletion exacerbates $CCl_4$ induced capillarization and basement membrane deposition (Xing et al., 2016). LXR also acts antagonistically on Hedgehog signaling (Hh) (Kim et al., 2009), while LSEC produce and respond to Hh ligands and use Hh signaling to regulate complex phenotypic changes that occur during capillarization. Moreover, inhibition of Hh using **cyclopamine** induced fenestration *in vitro* (Xie et al., 2012a).

**Plasmalemma vesicle-associated protein** (PLVAP) is associated with angiogenesis and vascular permeability, with less expression in barrier endothelium, and its expression is stimulated by VEGF (Bosma et al., 2018). PLVAP was found to be associated with a normally fenestrated phenotype, while PLVAP deficient mice present extremely low porosity and accumulation of collagen in the space of Disse (Herrnberger et al., 2014). Auvinen et al. (2019) found that there was no difference in number of fenestrations in PLVAP–/– mice, though their data shows greater variability in the knockouts. Both studies used SEM of tissue blocks for quantitative analysis of fenestrations. The difference may relate to the methods used to attain the knockouts raising the question of either knockouts being too broad/non-specific or insufficient. PLVAP mutations are associated with loss of fenestration diaphragms in other tissues (such as small intestine) (Elkadri et al., 2015).

## Lab Tools and Experimental Models

### Experimental Animal Models for the Study of LSEC Fenestrations

Liver sinusoidal endothelial cells are the first line of defense in the liver and alterations in LSEC play a crucial role in the development of many liver diseases such as fibrosis, cirrhosis, or cancer (Gracia-Sancho et al., 2021) as well as in the age-related conditions (Hunt et al., 2018). To better understand this role, many animal models have been used. Challenge with certain drugs can mimic the development of these diseases and reduce the time and/or costs compared to waiting for them to spontaneously occur in animals (**Table 3**). Although the exact mechanism of action of many of these drugs is not known, the outcome is similar enough to study and propose possible treatments.

Cirrhosis is a pathological liver state characterized by abnormalities in hepatic architecture such as loss of fenestrations

**TABLE 3 |** Experimental models and lab tools affecting LSEC fenestrations.

| | Fenestration diameter | Porosity | Fenestration frequency | References | Methods |
|---|---|---|---|---|---|
| **Cytoskeleton disruptors** | | | | | |
| Cytochalasin B | 0/+ | +++ | +++ | Braet et al., 1996a,b,c | a/b AFM, SEM, *in vitro* c SEM, TEM, *in vitro* |
| | | | | Steffan et al., 1987 | SEM, TEM, *in vitro* SEM, *in vivo* |
| | | | | Braet et al., 1995a | TEM, *in vitro* |
| | | | | Zapotoczny et al., 2017b, 2019b | AFM, *in vitro* live |
| | | | | Spector et al., 1999 | FL, SEM, TEM, *in vitro* |
| | | | | Oda et al., 1993 | SEM, TEM, *in vitro* |
| | | | | Van Der Smissen et al., 1986 | TEM, *in vitro*, *in vivo* |
| | | | | Steffan et al., 1986 | SEM, *in vivo* |
| | | | | Kalle et al., 1997 | AFM, *in vitro* |
| Cytochalasin D | 0/- | + | + | Svistounov et al., 2012; Hunt et al., 2019 | SEM, *in vitro* |
| Dihydrohalichondramide | - | nd | ++ | Braet et al., 2002 | SEM, *in vitro* |
| Halihondramide | - | nd | ++ | Braet et al., 2002 | SEM, *in vitro* |
| Jasplakinolide | - | nd | + | Zapotoczny et al., 2019b | AFM, *in vitro* live |
| | | | | Braet et al., 1998 | SEM, TEM, *in vitro* |
| | | | | Spector et al., 1999 | FL, *in vitro* |
| Latrunculin A | 0 | nd | ++ | Braet et al., 1996a | SEM, TEM, *in vitro* |
| | | | | Spector et al., 1999 | FL, *in vitro* |
| | | | | Braet et al., 1997 | SEM, *in vitro* |
| Misakinolide | - | nd | ++ | Braet et al., 1998, 1999; Spector et al., 1999 | SEM, TEM, *in vitro* |
| Swinholide A | - - | nd | +++ | Braet et al., 1998, 1999; Spector et al., 1999 | SEM, TEM, *in vitro* |
| **Disease models** | | | | | |
| Dimethyl nitrosamine (DMN) | - | - - | nd | Fraser et al., 1991, 1995b; Rogers et al., 1992; Tamba-Lebbie et al., 1993 | SEM, *in vivo* |
| Endotoxin/LPS | -/G | - -/0 | - | Dobbs et al., 1994; Fraser et al., 1995b | SEM, *in vivo* |
| | | | | Frenzel et al., 1977; Ito et al., 2006a | SEM, TEM, *in vivo* |
| | | | | Sasaoki et al., 1995 | SEM, *in vitro* |
| Galactosamine + endotoxin | G | nd | - | Ito et al., 2006a | SEM, TEM, *in vivo* |
| Galactosamine + endotoxin + matrix metaloproteinase | 0 | nd | 0 | Ito et al., 2006a | SEM, TEM, *in vivo* |
| Monocrotaline | G | nd | - - | DeLeve et al., 1999 | SEM, TEM, *in vivo* |
| | | | | DeLeve et al., 2003a,b | SEM, *in vivo* |
| Monocrotaline + V-PYRRO/NO | 0 | 0 | nd | DeLeve et al., 2003b | SEM, *in vivo* |
| Poloxamer 407 | nd | nd | - - | Cogger et al., 2006 | SEM, TEM, *in vitro*, *in vivo* |
| Pyocyanin | nd | - - | nd | Cheluvappa et al., 2007 | SEM, *in vitro* |
| Thioacetamide (TAA) | - | - - | nd | Mori et al., 1993a,b | SEM, TEM, *in vivo* |
| | | | | Xie et al., 2012b | SEM, *in vivo* |
| **Other** | | | | | |
| Superoxide anion (SOA) and nitric oxide NO | G | nd | - | Deaciuc et al., 1999 | SEM, TEM, *in vivo* |
| 7 keto cholesterol (7KC) | + | + | + | Svistounov et al., 2012; Hunt et al., 2019 | SEM, *in vitro* |
| Antimycin A | nd | - | - - - | Zapotoczny et al., 2017b | AFM, *in vitro* live |
| | | | | Braet et al., 2003 | SEM, TEM, *in vitro* |
| Arsenic | nd | - - | nd | Straub et al., 2008 | SEM, TEM, *in vitro*, *in vivo* |
| C3 transferase | + | + | nd | Yokomori et al., 2004 | SEM, TEM, *in vitro* |

*(Continued)*

**TABLE 3 |** (Continued)

| | Fenestration diameter | Porosity | Fenestration frequency | References | Methods |
|---|---|---|---|---|---|
| Calcium ionophore | - | nd | 0 | Zapotoczny et al., 2019a | AFM, *in vitro* |
| | | | | Oda et al., 1993 | SEM, TEM, *in vivo* |
| Calmodulin agonist w7 | + | nd | nd | Oda et al., 1993 | SEM, TEM, *in vitro* |
| Cyclopamine | nd | + | nd | Xie et al., 2012a | SEM, *in vitro* |
| Diamide | nd | nd | - - - | Zapotoczny et al., 2019a | AFM, *in vitro* live |
| Hydrogen peroxide | +/G | - -/+ | - | Cogger et al., 2001 | SEM, TEM, *in vivo* |
| | | | | Straub et al., 2008 | SEM, TEM, *in vitro*, *in vivo* |
| Iodoacetic acid | nd | nd | + | Zapotoczny et al., 2019a | AFM, *in vitro* live |
| Lysophosphatic acid (LPA) | - | nd | - - | Yokomori et al., 2004 | SEM, TEM, *in vitro* |
| Phorbol myristate acetate (PMA) | 0 | nd | - | de Zanger et al., 1997 | SEM, *in vitro* |
| *S*-nitroso-*N*-acetyl penicillamine (SNAP) | G | nd | 0 | Deaciuc et al., 1999 | SEM, TEM, *in vivo* |
| Staurosporine | 0 | nd | - | de Zanger et al., 1997 | SEM, *in vitro* |
| Tert-butyl hydroperoxide | G | + | 0 | Cogger et al., 2004 | SEM, TEM, *in vitro*, *in vivo* |
| Triton x100 | 0 | - - | nd | Svistounov et al., 2012 | SEM, *in vitro* |
| Trombospondin 1 | nd | - - | - - | Venkatraman and Tucker-Kellogg, 2013 | SEM, *in vitro* |

*"0," no change; G, gaps; nd, no data; increase: "+," <50%; "++," 50–100%; "+++," >100%; decrease: "-," <50%; "- -," >50%; "- - -," defenestration.*

(defenestration) and the build-up of basement membrane formed from collagen deposition in the space of Disse. Interestingly, the first stages of capillarization and defenestration was reported to be reversible prior to the deposition of collagen and formation of a basement membrane which indicates progression from fibrosis to cirrhosis (Xie et al., 2012b). Drugs such as **dimethyl nitrosamine (DMN)** or **thioacetamide (TAA**) are used to induce cirrhotic morphology in LSEC in animal models. Chronic admission of DMN (Fraser et al., 1991; Tamba-Lebbie et al., 1993) and TAA (Mori et al., 1993b; Xie et al., 2012b) was shown to lead to the loss of fenestrations, however the precise mechanism(s) behind this remains unknown. It was suggested that soluble guanine cyclase (sGC) is a crucial element of signaling necessary to maintain fenestrated LSEC morphology. sGC activation normalizes LSEC phenotype and completely prevents progression of fibrosis despite ongoing TAA exposure, so the limiting defect responsible for capillarization in this model of cirrhosis was in the NO/sGC/cGMP pathway (Xie et al., 2012b). Defenestration is an important step not only in cirrhosis and fibrosis but also with aging and its development and has an impact on the whole organism. Lack of filtration of chylomicrons and chylomicron remnants leads to hyperlipidemia (Rogers et al., 1992). Cogger et al. (2006) showed that **poloxamer 407**, a synthetic surfactant causes dramatic defenestration and massive hyperlipidemia. This finding suggests a direct role of LSEC porosity in the lipid clearance in the liver.

**Monocrotaline** has been used to a model hepatic veno-occlusive disease (DeLeve et al., 1999) and sinusoidal obstruction syndrome (SOS) (DeLeve et al., 2003a,b). Toxic effects were observed only in LSEC but not in hepatocytes nor in other parts of the endothelium. LSEC metabolize monocrotaline by conjugation to glutathione and detoxify to pyrrolic metabolite. It is believed to be a stable reproducible model resulting in a decreased number of fenestrations, gap formation and discontinuous sinusoid occurrence (DeLeve et al., 1999). It is an important reminder that LSEC also can metabolize drugs and it is not only the hepatocytes that have this function in the liver.

**Galactosamine**, together with **endotoxin** or **TNF**, causes gap formation in the sinusoids and can be used to study the neutrophil extravasation in the acute inflammatory tissue injury (Ito et al., 2006a). It was shown that inhibition of **matrix metalloproteinases**, which are involved in gap formation, reduces the neutrophil accumulation in the sinusoids. **Bacterial endotoxin** alone plays a role in the pathogenesis of cirrhosis, decreasing both number and diameter of fenestrations (Dobbs et al., 1994). Other bacterial toxins, such as **pyocyanin** or **LPS**, are used in studies of post-transplantation complications such as sepsis or ischemia-reperfusion injury. **Pyocyanin** treatment decreases porosity by its effects on the frequency of fenestrations and can be prevented by addition of catalase. This result suggests that the mechanism involves hydrogen peroxide–induced oxidative stress (Cheluvappa et al., 2007).

Another bacterial toxin, ***Clostridium botulinum* C3-like transferase (C3-transferase**), together with **lysophosphatic acid (LPA)** was tested in a study from 2004. C3-transferase is a rho inhibitor, while LPA is a rho stimulator. Rho was found to be an important regulator of the actin cytoskeleton and was therefore tested for its influence on fenestration and LSEC in general. The *in vitro* experiments on rat LSEC showed dilation and fusion of fenestrations after treatment with C3-transferase, while contraction occurred when the cells were treated with LPA. Additionally LPA caused an increase in F-actin stress fiber and actin microfilaments, while C3-transferase treatment showed the opposite (Yokomori et al., 2004).

Several models of experimental liver injury show similar morphological alterations, including gaps and ruptured sinusoids. Deaciuc et al. (1999) showed that these early changes can be mediated by the free radical species. The *in vitro* treatment

of rat LSEC with **superoxide anion** or **nitric oxide** resemble the observations from *in vivo* experiments with various hepatotoxins. Treatment with **hydrogen peroxide** also increased fenestration diameter and decreased fenestration number (Cogger et al., 2001). High porosity values can be misleading in the studies where gap formation is observed so measurement of all three morphology parameters should be considered. Straub et al. (2008) presented that effect of low doses of **arsenic**, mimicking water contamination levels, also act through reactive oxygen species (ROS) generated by NADPH oxidase (NOX). This mechanism was confirmed by the protective (against arsenite) results from NOX deficient mice and use of NOX inhibitors.

### Cytoskeleton Disruptors

Numerous agents acting on the actin cytoskeleton have significant effects on fenestration (**Table 3**). Two main groups include marine sponge- and mushroom-derived toxins. Relatively well-known mechanisms of action of these toxins allowed the study of the link between actin cytoskeleton and fenestrae. An extensive chapter from Braet et al. (2008), provides an overview on the *in vitro* effects of actin binding agents such as **cytochalasin B, latrunculin A, jasplakinolide A, swinholide A, misakinolide A, halichondramide, and dihydrohalichondramide**. Despite different mechanisms of promoting/inhibiting actin polymerization or fiber stabilization, all drugs result in an increase of fenestration number. The most surprising finding is the effects of jasplakinolide which promotes polymerization and stabilization of actin in other cells, but in LSEC no such effect was shown. Instead, the loss of fibers and accumulation of actin in single spots occurs within minutes of jasplakinolide treatment (Spector et al., 1999). These structures, described as 'actin dots,' are not fully understood, but they resemble recently described actin asters which may be connected with lipid raft reorganization (Fritzsche et al., 2017). There is an ongoing discussion about the specificity of those agents for actin. For example, cytochalasin B (but not D) was shown to influence transport of glucose across cell membranes and its overall effect can be influenced by changes in glycolysis and metabolism (Kapoor et al., 2016). **Iodoacetic acid** acts on both actin and spectrin and was shown to decrease stress filament formation. Moreover, it caused an increase in porosity and rapid opening and closing of fenestrations (Zapotoczny et al., 2019a). Nevertheless, agents acting on the actin cytoskeleton remain the most important tools for studying fenestration structure and dynamics.

### Other Agents Affecting Fenestrations

Svistounov et al. (2012) emphasized the importance of lipid membrane stability and lipid rafts on LSEC morphology. Surfactants such as **Triton X100** or **poloxamer** showed destabilization of the cell membrane and promotion of lipid raft formation which resulted in a decrease or even complete ablation of fenestrations. Moreover, the reduction of lipid raft formation by **7 keto-cholesterol (7KC)** increased the number of fenestrations showing the connection between fenestration structure, actin and cell membrane (Hunt et al., 2019).

**Thrombospondin 1** (TSP) is a matrix glycoprotein with pro-fibrotic effects. In a study from 2013 (Venkatraman and Tucker-Kellogg, 2013) it was shown to cause dose-dependent defenestration in LSECs at 100 ng/mL. The authors additionally showed that the CD47-binding fragment of TSP1, p4N1 – which has anti-angiogenic effects in endothelial cells, also induces defenestration in LSECs.

The influence of **phorbol myristate acetate (PMA)**, a protein-kinase-C (PKC) activator and **staurosporine**, a PKC inhibitor, on LSEC have been examined by de Zanger et al. (1997). The *in vitro* treatment of rat cells for 2–7 days resulted in a decrease in porosity, due to the decrease in fenestration number without any observable change in fenestration diameter, when treated with PMA. However, despite the decrease in porosity, PMA improves LSEC cultures in terms of viability and purit, and fenestrated morphology was maintained after 7 days (de Zanger et al., 1997). Treatment with staurosporine or PMA and staurosporine showed enlarged fenestrations, gap formation and a decrease in porosity. The authors concluded that PMA acts on LSEC through PKC based on the staurosporine treatment neutralizing the PMA treatment effects.

Deaciuc et al. (1999) tested rat livers challenged with superoxide anion [***S*-nitroso-*N*-acetyl penicillamine (SNAP)**] and nitric oxide [xanthine oxidase plus hypoxanthine (XO + HX)] generating substances. They theorized that early morphological LSEC alterations associated with liver injury are influenced by free radical species. When they perfused the rat livers with SNAP, they found a suppression of hyaluronan uptake (a test of LSEC endocytosis capacity) and the formation/creation of large gaps in LSEC morphology, sometimes instead of sieve plates, and sometimes together with fenestrations present in sieve plates.

## MECHANISMS

As discussed above, a variety of agents have been tested so far showing their effect on fenestrae. Some of the agents changed the number of fenestrations, while others alter their diameters or distribution (gathered in sieve plates or individual fenestrations), including the formation of gaps. However, the clear understanding of why individual drugs have their effects on LSEC is still lacking. The main reason is that many drugs have cross-effects at the cellular level, affecting more than one cellular mechanism/pathway, including the rearrangement of cytoskeleton. Therefore, it is challenging to predict how a drug will work on LSEC fenestrations.

A thorough analysis of the effects of a variety of agents changing porosity, fenestration frequency, and fenestration diameters (including gap formation) resulted in four different hypotheses. These independent but overlapping ideas describe the possible mechanisms behind fenestration structure and dynamics.

(I) *Actin (de)polymerization regulates the number of fenestrations* (Braet et al., 1996b; Spector et al., 1999; Braet and Wisse, 2002; Mönkemöller et al., 2015). The hypothesis was discussed in Braet et al. (1995a), Braet et al. (1996b) and has been developed over the years. It was presented that the cytoskeleton plays a crucial role in the porosity

of LSEC. Fenestrae-associated cytoskeleton rings (FACR) surround each fenestration and sieve plate-associated cytoskeleton surround sieve plates (Braet et al., 1995b). The application of actin (de)polymerization targeting drugs revealed the direct connection between actin cytoskeleton and fenestration number in LSEC (Spector et al., 1999; Carpenter et al., 2005). However, the disruption of actin does not destroy fenestration structure, which indicated the complex structure of FACR. Later it was reported that actin filaments surround each fenestration within a sieve plate (Mönkemöller et al., 2015).

(II) *Calcium ions regulate the diameter of fenestrations*. This second hypothesis was summed up in 2002 (Braet and Wisse, 2002). It is mainly based on the research of Oda and Yokomori presenting the role of calcium/calmodulin/actomyosin in the contractility of fenestration diameters (Oda et al., 1990; Yokomori et al., 2004). The regulation of myosin light chain (MLC) phosphorylation occurs via calcium-calmodulin signaling. Further it was suggested that MLC kinase and phosphatase may exert different effects on cell morphology (Yokomori et al., 2004).

(III) *Regulation of fenestrations depends on lipid rafts*. The sieve-raft hypothesis assumes that fenestrations are formed in the flat areas of the cell periphery, in between lipid rafts, where the cell membrane is more flexible and more prone to shape changes (Svistounov et al., 2012). Also, other ways in which lipid rafts can be connected with fenestration were proposed, such as influence on signal transduction or indirect regulation of some signaling pathways.

(IV) *Spectrin is involved in the open versus closed state of fenestration*. The hypothesis decouples the direct actin regulation from the number of fenestrations. Instead, the interplay between the membrane scaffold and actin cytoskeleton is responsible for the opening of the fenestration within the actin ring (Zapotoczny et al., 2019a).

All the above hypotheses do not exclude each other and only emphasize how complicated the mechanisms regulating the number, shape, and size of fenestrations can be. In the following subsections we will focus on the physiological regulation of number and size of fenestrations, apart from the direct (often toxic) effect of actin disturbing drugs (described above). The analysis of different agents acting on LSEC fenestrations leads to the conclusion that the phosphorylation of myosin light chain (MLC) is the core of various pathways regulating actin (de)polymerization. Calcium dependent and independent activation (phosphorylation) of MLC and release of actin binding proteins (such as tropomodulin, tropomyosin, caldesmon) leads to contraction of fenestrations and decrease in the number of fenestrations, while MLC dephosphorylation leads to the relaxation of MLC and promotes more fenestrated morphology of LSEC. The local balances regulating the levels of calcium, ROS, or NO in different parts of the cell ensure active control over the dynamics of fenestrated LSEC. The regulation covers the (de)activation of membrane proteins which may affect actin association to the membrane. Finally, the oxidative state of membrane cytoskeleton and lipid rafts distribution are additionally (passively or actively) involved in this regulation.

## Cytoskeleton

SEM and TEM allowed visualization of the fenestrae-associated cytoskeleton rings (FACR) in LSEC (Braet et al., 1996b). Preparations of "ghost" cells, after removing cell membrane with detergent, revealed a network of filaments associated with sieve plates surrounded by thicker filaments. Precise identification was not possible, but the high resolution of those techniques allowed diameter measurements suggesting a mesh of actin fibers surrounded by microtubules. The gap in the chemical information has been filled with super resolution fluorescence microscopy. Mönkemöller et al. (2015) showed the first direct correlation between the localization of cell membrane and actin around fenestration, using SIM. Recently, FACR structures could be also visualized in high resolution using AFM and dSTORM (Zapotoczny et al., 2017b, 2019a). It was also presented that the complete actin ring is necessary to form an open pore within a FACR (Zapotoczny et al., 2019a).

Cytoskeleton remodeling that influences the number of fenestrations was demonstrated for live LSEC. During the first hours after isolation LSEC spread on the substrate, opening and closing individual fenestrations and whole sieve plates. It indicated that fenestrations are not preserved from the *in vivo* to the *in vitro* state and their formation and closing is dynamic as previously suggested (Braet and Wisse, 2012). With time, the dynamics of fenestrations was shown to be slower (Zapotoczny et al., 2020). Still, fenestrations in isolated LSEC were shown to freely migrate several micrometers, and changing their diameter up to 200% during their $\sim$ 20 min lifespan.

Interesting labyrinth like structures have been observed *in vitro* in the proximity of the perinuclear area of LSEC (Braet et al., 2009). Some fenestrations form three dimensional multi-folded tunnels that are not always passing through the cell which contradicts the sieving role of LSEC. One possible explanation could be that these structures are caused by the cell isolation process because they have not been observed *in vivo* (in tissue samples). After digestion of the liver with Liberase/collagenase cells are detached from each other, perhaps disrupting parts of their cytoskeleton in a way that can be beyond repair after reattachment *in vitro*. Another explanation assumes that microfilament-disruption induces translocation of pre-existing three-dimensional organized fenestrae forming centers (FFCs) from the perinuclear area toward the peripheral cytoplasm (Braet et al., 1998, 2007). Recently, the formation of FFC was shown in live LSEC. It was confirmed that FFC are involved in the rapid increase in fenestration number, both in control and drug treated LSEC.

The importance of the actin cytoskeleton and the structure of FACR was confirmed by the dramatic effects of any agent directly affecting actin. Actin disruptors (see **Table 3** and **Figure 3**) were shown to rapidly induce the formation of new fenestrations (up to 300% porosity increase in 30 min by cytochalasin B) despite different mechanisms of actin depolymerization (Steffan et al., 1987; Zapotoczny et al., 2017b).

Other drugs that indirectly cause actin depolymerization, such as iodoacetic acid, metformin or sildenafil, also resulted in the increase in fenestration number (Hunt et al., 2019; Zapotoczny et al., 2019a). Altogether, agents acting on actin cytoskeleton remain the most important tools in studying fenestration structure and dynamics.

Understanding the mode of action of actin disturbing agents may help us reveal fenestration structure. Actin fibers are regulated by a set of proteins such as profilin, gelsolin, or cofilin that create the dynamic, out-of-equilibrium state. Every actin-binding protein, regardless of the location of its actin-binding site, influences the adenine nucleotide exchange rate of actin and the ratio of G (monomer/globular) and F (polymerized/filamentous) actin (**Figure 3**). Control over that process is maintained by many signaling pathways allowing LSEC to adjust the morphology according to internal and external stimuli. Actin disrupting agents act similarly to those controlling proteins. However, they lack control or feedback loop systems therefore result in rapid and dramatic changes. The importance of the controlled signaling is especially visible in prolonged *in vitro* LSEC culture where changes in cytoskeleton, such as stress fiber formation and fenestration disappearance, occur (Yokomori et al., 2004). However, the direct relationship between the actin polymerization into the thick stress fibers and the decrease in the number of fenestrations needs to be evaluated.

In fact, actin is the only demonstrated protein that was validated to have a direct impact on the number of fenestrations. Therefore, we discuss the various signaling pathways leading to actin and actin related proteins and the ways to affect them to observe the desired effect on fenestrations in the next section.

## MLC Phosphorylation – The Core of the Fenestration's Regulation

Myosins convert ATP to create a mechanical force on actin. Created tension in actomyosin cytoskeleton is necessary for number of cellular processes, including cell motility, cytokinesis and intracellular trafficking (Brito and Sousa, 2020). The myosins contain a neck region allowing to bind myosin light chain (MLC) domains, which are regulated by the phosphorylation and dephosphorylation via MLCK and MLCP respectively. In its phosphorylated/active form, MLC results in activation of ATP dependent myosin heavy chain binding to f-actin, which creates an active contractile force. With 30 classes of molecular motors in myosin superfamily regulating variety of cellular processes (Brito and Sousa, 2020) several reports have been dedicated to the role of MLC in the regulation of fenestration diameters. In the following subsections we focused on the cellular machinery involved in the regulation of MLC phosphorylation via calcium, NO, and ROS pathways.

## Lipid Rafts

The existence and role of lipid rafts has caused divisions in the scientific community in recent years and during The Keystone Symposium on Lipid Rafts and Cell Function (2006) the following definition was adopted: "Membrane rafts are small (10–200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein–lipid interactions." The role of lipid rafts in fenestrations structure and dynamics was studied only recently (Svistounov et al., 2012) and then the hypothesis of sieve-raft regulation of fenestrations was proposed by Cogger et al. (2013). Visualization with SIM revealed that rafts are not present inside sieve plates but rather surround them in an inverse distribution (Svistounov et al., 2012). Fenestrations are formed in the flat, non-raft lipid-disordered regions and are prone to changes in raft organization. 7 keto cholesterol (7KC) increases lipid ordered, non-raft regions and thus promotes fenestration formation while detergent Triton X-100 increases the relative area of raft rich regions and decreases fenestration number (Svistounov et al., 2012; Hunt et al., 2018) (causing complete defenestration at high Triton X-100 concentrations). High doses of 7KC caused gap formation and retraction of cell membrane, which can be explained by deficits in cell membranes after depletion of rafts. Another detergent, poloxamer 407, was also reported to elicit massive defenestration of LSEC (Cogger et al., 2006). Interestingly, pre-treatment with Triton X-100 (increases rafts) abrogated the effect of cytochalasin D and no increase in porosity was observed (Svistounov et al., 2012). This result elucidates the tight connection between rafts and actin cytoskeleton in fenestration structure and/or dynamics. However, it was reported that the lipid rafts in biological membranes induced by detergents may not fully resemble the normal functional rafts (Heerklotz, 2002).

Rafts are enriched in sphingolipids and cholesterol which engenders membrane stability and provides a platform for many membrane proteins that may contribute to their connection to the actin cytoskeleton (Viola and Gupta, 2007). The anchoring of actin to the lipid rafts was suggested to be realized through the FERM domain of ERM proteins and talin (Chichili and Rodgers, 2009), as well as adducin (Yang et al., 2018) and spectrin (Ciana et al., 2011). Functional rafts may not be steady-state phenomena; they might form, grow, cluster or break up, shrink, and vanish according to functional requirements, regulated by rather subtle changes in the activity (disordering or ordering) of membrane compounds (Heerklotz, 2002). These properties might be connected with the dynamic nature of fenestrations and LSEC's ability to rapidly respond via morphology changes. The amount of lipid rafts may also have an indirect effect on fenestrations, through interactions independent of actin. It has been reported that ABC transporters, which decrease intracellular cGMP levels by its efflux, work less efficiently out of raft regions (Klappe et al., 2009). cGMP is an important signaling molecule that acts on fenestrations through PKG, decreasing intracellular calcium and promoting relaxation, both of which are connected with growing fenestration number. Lipid rafts may also affect many signal transduction pathways in the cell by serving as platforms to bring receptors into proximity with activating kinases, scaffolding proteins, and adaptor molecules that are constituent residents of lipid rafts (Rauch and Fackler, 2007).

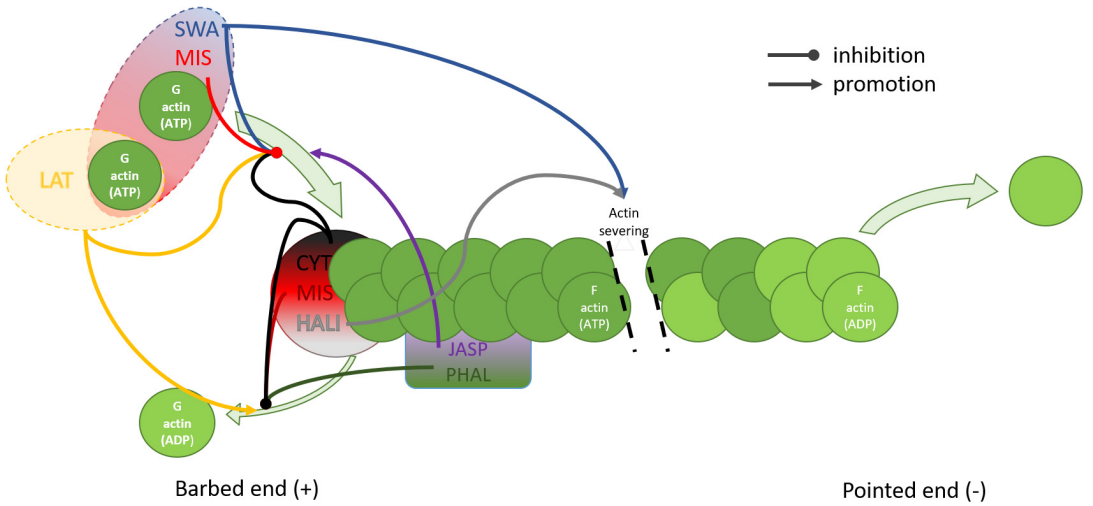


**FIGURE 3 |** Schematic representation of the effects of actin disrupting drugs on actin filaments. Depolymerization of the barbed end of the actin filament is inhibited by CYT, MIS, and HALI which cap the barbed end, and by PHAL and JASP which attach from the side, additionally stabilizing the fiber. Latrunculin promotes depolymerization by specific sequestration of monomeric actin. Polymerization is stimulated by JASP which also binds competitively to PHAL. Barbed end polymerization is inhibited by CYT, LAT, SWA, and MIS. Both MIS and SWA bind two actin monomers, however only MIS caps the barbed end. HALI and SWA stimulate severing of the actin filament. CYT, cytochalasin; HALI, halihondramide; JASP, jasplakinolide; LAT, latrunculin; MIS, misakinolide; PHAL, phalloidin; SWA, swinholide.

## Spectrin

It was reported that only completely closed FACR structures contained fenestrations in the open state (Zapotoczny et al., 2019a). It was proposed that spectrin arranges actin to form a ring-like structure. Although the actin cytoskeleton is important part of fenestration structure, the membrane scaffold has a role in the regulation of opening of fenestration within FACR. In the spectrin-actin hypothesis, fenestrations can be opened if the cell height does not exceed 300–400 nm, which is double the length of the spectrin unit (Zapotoczny et al., 2019a). The proposed mechanism is based on the observation of both open and closed fenestrations within actin rings in live LSEC *in vitro*. The switch between the open and closed state was pharmacologically induced. The actin-spectrin complexes are strong enough to allow migration of the individual fenestrations across the cell membrane. Moreover, it can explain, why actin depolymerizing agents induce new fenestrations: spectrin can arrange short actin fibers to form ring like structures, and decreased cell height allows spectrin units to bind, forming new FACRs. In 2020, the role of actin/fodrin (non-erythroidal spectrin) was reported to be required in fenestration biogenesis in the endothelioma cell line bEND5, in which fenestrations can be induced pharmacologically (Ju et al., 2020). Authors showed a close association between beta actin and spectrin. Moreover, they reported that knockout of alpha spectrin resulted in 10-fold decrease in the number of fenestrations. Nevertheless, despite the increasing interest in this membrane cytoskeletal protein the knowledge of membrane skeleton regulation in endothelial cells is poorly understood.

## Regulation *via* $Ca^{2+}$

The role of calcium in the regulation of fenestration diameters was discussed by Braet and Wisse (2002). The serotonin induced influx of calcium was described to cause calcium-calmodulin dependent phosphorylation of MLCK decreasing the size of fenestrae, denoted as contraction. The reverse effect remained as speculation. Later, Yokomori et al. (2004) summarized that calcium influx affected not only MLCK, but also Rho activity. Thus, calcium can affect both MLCK and ROCK dependent phosphorylation of MLC. The authors presented results of LPA and C3 transferase, causing fenestration closing and dilating respectively, indicating that they act through MLC phosphorylation. In the **Figure 4** we extended the possible regulation of MLC phosphorylation, based on the current state of knowledge. MLC is activated by the calcium mediated phosphorylation via myosin light chain kinase (MLCK) (Rigor et al., 2013). The activity of MLCK is increased by $Ca^{2+}$-calmodulin binding and by phosphorylation by protein kinase C (PKC). PKC can also further promote MLC phosphorylation by inhibition of MLCP, however, this pathway was not confirmed in endothelium (Somlyo and Somlyo, 2000). The activation of MLCK can be hampered by the cAMP dependent kinase – protein kinase A (PKA). PKA binds to the similar region of MLCK to the $Ca^{2+}$-calmodulin complex binding domain, hampering calcium dependent MLC phosphorylation. However, the activation of MLC is not sufficient to create a contractile force of the actomyosin complex. The actin binding proteins ensure additional control. Actin is stabilized by e.g., tropomyosin, tropomodulin, caldesmon, or calpain. The release of these proteins from actin is controlled in a calcium-concentration-dependent manner, allowing myosin to reach actin (Hepler, 2016). Moreover, the activation of actin polymerization processes, e.g., by gelsolin, profilin or cofilin is also calcium dependent and results in an increase in actin polymerization. The calcium level, regulated by calcium membrane channels and pumps or

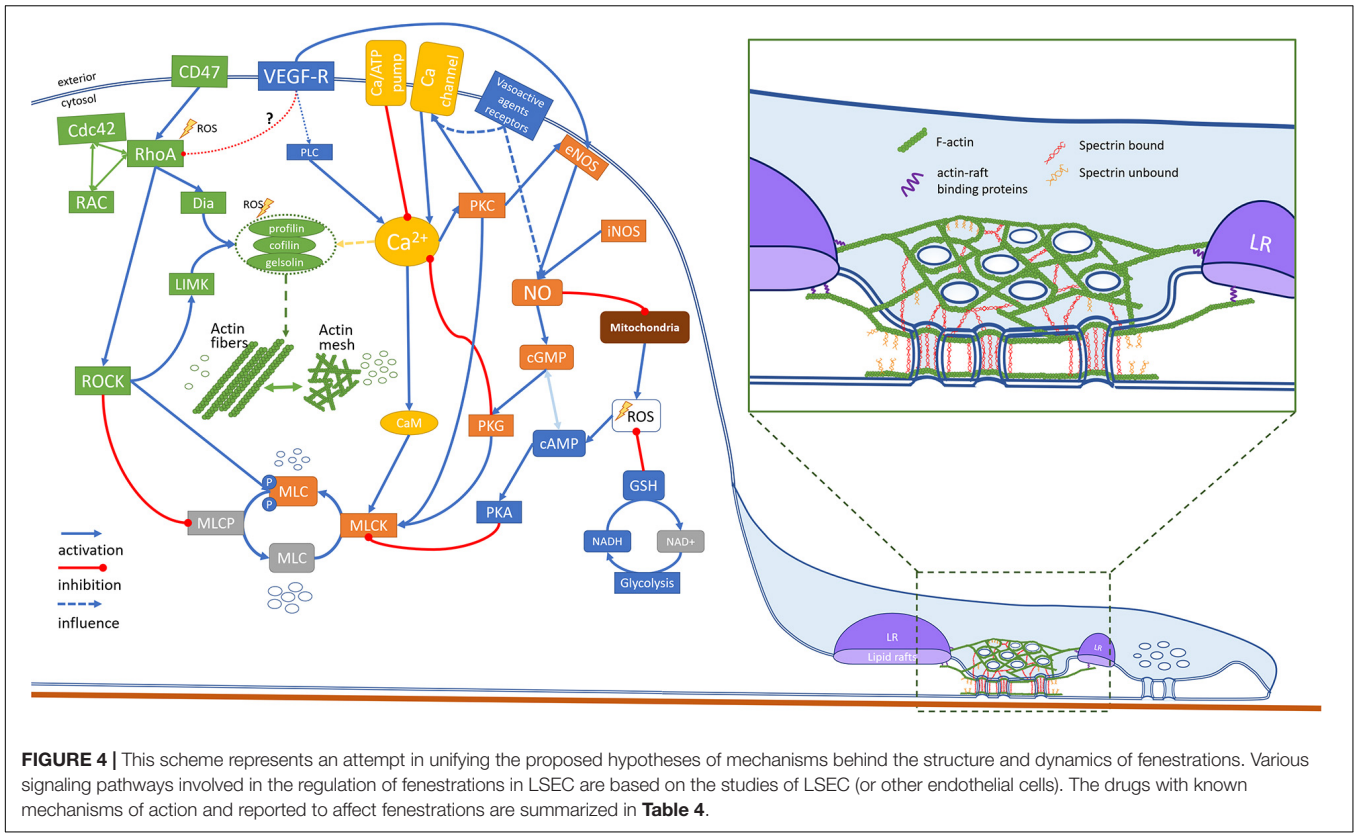


**FIGURE 4 |** This scheme represents an attempt in unifying the proposed hypotheses of mechanisms behind the structure and dynamics of fenestrations. Various signaling pathways involved in the regulation of fenestrations in LSEC are based on the studies of LSEC (or other endothelial cells). The drugs with known mechanisms of action and reported to affect fenestrations are summarized in **Table 4**.

by endoplasmic reticulum release, causes a cascade of cellular mechanisms driving local changes in the cytoskeleton. These changes vary in different cells and the details of these processes is beyond the scope of this review. The contraction of actomyosin is permanent. It means that it must be actively undone to ensure actomyosin relaxation. The balance of (de)phosphorylation of MLC is maintained by MLC phosphatase (MLCP). The enzyme activity is independent of the calcium plasma concentration (Álvarez-Santos et al., 2020). In addition to the role in the dephosphorylation of MLC, it exhibits phosphatase activity toward other proteins, such as ankyrin, adducin, Tau, merlin, calcineurin-A, interleukin-16, Rb, moezin, and ezrin (Kiss et al., 2019). Inhibition of MLCP (MYPT1 complex) by activation of the RhoA/ROCK pathway, results in indirect increase in the level of phosphorylated MLC and an increase in/of the contractile forces. PKA, PKG, and PKC also cause phosphorylation of MLCP. However, a recent study showed that in contrast to the RhoA/ROCK pathway, PKG- induced phosphorylation has no effect on MLCP activity (MacDonald and Walsh, 2018). It needs to be emphasized that the phosphorylation of MLC is connected to the formation of fibrous actin (via activation of actin nucleation proteins – e.g., gelsolin, profilin, cofilin, as mentioned) and vice versa. It was suggested that actin polymerization is necessary for force development (Mehta and Gunst, 1999). Therefore, the actin relaxation/contraction state is to some extent connected with the (de)polymerization of actin. The effects of certain drugs on fenestrations may be a sum of both.

## Regulation *via* NO

Nitric oxide is one of the most important signaling molecules in endothelial cells and plays a crucial role in the maintenance of fenestrations in LSEC (DeLeve, 2015). NO stimulates sGC synthase and thus increases the cGMP level which then starts a cascade of signaling. cGMP stimulates the efflux of intracellular calcium into endoplasmic reticulum storage which reduces activation of MLCK through calmodulin. There are also suggestions that cGMP in microvascular endothelium can act through PKG to activate MLCP leading to further dephosphorylation of MLC (Rigor et al., 2013), but this mechanism was shown only in vascular smooth muscle cells. As described above, we propose that inactivation of MLCK together with a decrease in $Ca^{2+}$ leads to actin relaxation, which results in the increase in fenestration diameter and/or number. There is also evidence of crosstalk between cGMP and cAMP levels which could further affect the MLC phosphorylation state (Chong et al., 2005). The exact mechanisms of action of NO on LSEC fenestration have not been described yet, however the cGMP/Ca pathway has been shown to be a part of VEGF induced NO production (Xie et al., 2012b; DeLeve, 2015). Two main sources of intracellular NO are synthases eNOS (activated among others by VEGF, endothelin, or estrogen) and iNOS (activated by cytokines during liver injuries). Both are responsible for LSEC phenotype maintenance as well as cell response to pathophysiological conditions (DeLeve et al., 2003b). The results of treatment with PMA — which activates PKC and can lead to increased NO production by eNOS — show a positive

**TABLE 4 |** Agents with known mechanism of action and their effects on LSEC fenestrations.

| Inhibitor | Target | Effect | References |
|---|---|---|---|
| C3 transferase | RhoA | FN ↑, D ↑ | Yokomori et al., 2004 |
| Simvastatin | CD47 | FN ↑, D ↑ | Hunt et al., 2019 |
| Y27635 | ROCK | FN ↑ | Venkatraman and Tucker-Kellogg, 2013 |
| W7 | Calmodulin | D ↑ | Oda et al., 1993 |
| 7 keto cholesterol | Lipid rafts | FN ↑, D ↑ | Svistounov et al., 2012 |
| Amlodipine | Ca channel | FN ↑ | Hunt et al., 2019 |
| **Promotor/activator** | **Target** | **Effect** | **References** |
| LPA | RhoA | D↓ | Yokomori et al., 2004 |
| Sildenafil<br>Amlodipine<br>TRAIL | cGMP | FN↑ | Hunt et al., 2019 |
| Phorbol myristate | PKC | FN↓ | de Zanger et al., 1997 |
| Thrombospondin | CD47 | Defenestration | Venkatraman and Tucker-Kellogg, 2013 |
| Simvastatin | NO | FN ↑, D ↓ | Venkatraman and Tucker-Kellogg, 2013; Hunt et al., 2019 |
| Serotonin | Ca channel | D↓ | Gatmaitan and Arias, 1993; Braet et al., 1995a |

*FN, fenestration number; D, fenestration diameter; ↑/↓, increase/decrease.*

effect on maintenance of LSEC morphology *in vitro* (de Zanger et al., 1997). The effect was confirmed by co-administration of staurosporine, which inhibits PKC.

The effect of NO is complex and involves many different pathways. Besides cGMP signaling, NO can (competitively to O2) bind to complex IV in mitochondria, blocking the electron transport chain which results in an increased ROS production (Moncada and Erusalimsky, 2002). NO can then combine with ROS creating highly reactive peroxynitrate $ONOO^-$. NO production by NOS is calcium dependent but at the same time NO contributes to changes in intracellular calcium. Those mechanisms seem to work as a feedback loop gently steering the cell response, especially since NO is not a stable molecule so its influence is restricted to areas local to its synthesis. In LSEC, NO is required for fenestration maintenance. However, it is not sufficient alone, and other NO independent pathways are necessary. It has been shown that, besides NO production stimulated by VEGF, NO-independent VEGF signaling is needed also (Xie et al., 2012b). We propose two possible mechanisms: in endothelial cells VEGF can act through its membrane receptor on PLC, followed release of the $Ca^{2+}$ from the endoplasmic reticulum (Rigor et al., 2013). Then, PKC enters a feedback loop of NO production leading to a decrease in $Ca^{2+}$. This would even further increase the NO production, but also would act as a balancing effect for calcium ions. NO can also induce protein *S*-nitrosilation, however it has been found not to affect fenestrations (Xie et al., 2012b). The other possibility is, reported in HUVEC, inhibition of Rho/ROCK pathway by VEGF receptors (Tagashira et al., 2018) which has been shown to play an important role in fenestration maintenance.

The cGMP pathway is a promising target for novel therapeutics for liver diseases and aging as restoration of cGMP levels can restore fenestrations in LSEC (Xie et al., 2012b). Drugs such as sildenafil influence cGMP by blocking its efflux by ABC transporters and degradation by phosphodiesterases (PDE) (Toque et al., 2008; Sager et al., 2012). Amlodipine, a blood pressure medication also affects fenestrations by acting through both cGMP and inhibition of $Ca^{2+}$ channels (Berkels et al., 2004). Another drug used for lowering blood lipid levels – simvastatin, promotes NO production directly via the Akt pathway and through inhibition of Rho GTPases (de Sotomayor and Andriantsitohaina, 2001).

## Regulation *via* ROS

There are many sources of ROS within the cell, such as the mitochondrial electron transport chain, NADPH and xanthine oxidase and, highly expressed in endothelium, eNOS when uncoupled (Widlansky and Gutterman, 2011; Jerkic and Letarte, 2015). ROS were initially considered mostly as cytotoxic, but recent reports summarize their positive regulatory roles both in physiological and pathological endothelium, reviewed in Widlansky and Gutterman (2011).

Recently the cytoprotective role of ROS through activation of autophagy signaling was shown in early ischemia injury (Bhogal et al., 2018). LSEC morphology is sensitive to ROS levels and many agents act through this mechanism, such as e.g., ethanol and acetaminophen causing the disappearance of fenestrations (Deaciuc et al., 1999). *In vivo* studies showed large gaps in LSEC caused by ROS, generated by xanthine oxidase and hypoxanthine suggesting destabilization of fenestrations which also prevent cells from closing those gaps (Deaciuc et al., 1999). Glutathione (GSH) is the main physiological countermeasure to free radicals such as ROS. Reducing agents such as NAC can reduce the depletion of GSH due to the presence of oxidative stress (Sun et al., 2014). The effect of ROS on fenestrations may come from different mechanisms based on the disturbance of the redox balance in the cell. Intracellularly, mitochondria are the main source of ROS while glycolysis is the main source of reducing agents such as GSH and NADH. Scavenging of ROS directly activates the Rho/ROCK signaling pathways (Popova et al., 2010) which may lead to promotion of stress fibers. By analogy, the reduction of ROS by antioxidants should lead to reduction of Rho/ROCK signaling, therefore promoting fenestration formation. This mechanism would explain the age-related defenestration associated with higher levels of ROS and reduced redox capabilities in the cells (Herrera et al., 2010).

In endothelial cells, ROS can act as a messenger molecule activating various signaling pathways. Besides the mitochondria, a second main ROS source are NAD(P)H oxidases which can be stimulated by various vasoactive agents (Griendling et al., 2000). It has been shown that LSEC morphology is sensitive to both vasodilators and vasoconstrictors, which was shown to increase and decrease the fenestration diameter respectively (**Table 2**). Moreover, LSEC lack underlying smooth muscles cells to emphasize the response to vasoconstrictors/dilators. There might exist more complicated cellular mechanisms in LSEC to compensate for this. Altogether, those findings suggest

that ROS may be part of signaling cascades activating redox-sensitive proteins.

## CONCLUSION

Drug clearance mediated by the liver is heavily dependent on the proper phenotype of LSEC, including the transport through fenestrations. Individual drugs and stimulants have been reported to influence the porosity of LSEC. Some drugs show beneficial effects on LSEC phenotype, potentially allowing re-opening fenestration ("re-fenestration") which could be of benefit in the elderly. The role of LSEC senescence and "anti-aging" senolytic drugs, with regard to porosity, warrants further study. However, the background of polypharmacy (regular daily consumption of 4 or more medicines) in much of the elderly population needs to be considered in the refenestration context. Within this review we highlighted the areas of research which will be particularly beneficial for both physicians and researchers. LSEC research is growing in recent years and the latest stage of our knowledge about fenestrations is now facilitated with novel microscopic techniques. These super-resolution methods will continue to improve, so it is appropriate for the field to simultaneously improve sample status, for example to examine living LSEC, or "wet" fixed preparations of LSEC or whole liver mounts instead of dehydrated cells. The substrate upon which LSEC are typically cultured also likely needs to be re-worked – tissue culture plastic is considerably stiffer than the LSEC's natural surroundings, so other softer gel-based substrates should be considered, such as those described by Guixé-Muntet et al. (2020). Ultimately, *in vivo* imaging of LSEC fenestrations *in situ* would be the ideal real-time test of refenestration therapies, but the challenges (e.g., movement due breathing and heart beat) for this type of technology are rather significant. That said, existing technologies should allow for comprehensive studies and better understanding of these unique structures, and how they work, in the coming years.

## AUTHOR CONTRIBUTIONS

KS, LK, and CH prepared the figures and tables. PM and BZ acquired the funding. All authors took part in conceptualization, analysis and writing of the manuscript, are responsible for all aspects of the manuscript and read and agreed to the submitted version of the manuscript.

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# Imbalanced Activation of Wnt-/β-Catenin-Signaling in Liver Endothelium Alters Normal Sinusoidal Differentiation

*Philipp-Sebastian Koch[1,2*†], Kajetan Sandorski[1,2†], Joschka Heil[1,2], Christian D. Schmid[1,2], Sina W. Kürschner[1,2], Johannes Hoffmann[1,2], Manuel Winkler[1,2], Theresa Staniczek[1,2], Carolina de la Torre[3], Carsten Sticht[3], Kai Schledzewski[1,2], Makoto Mark Taketo[4], Felix A. Trogisch[2,5], Joerg Heineke[2,5], Cyrill Géraud[1,2,6], Sergij Goerdt[1,2] and Victor Olsavszky[1,2]*

[1] Department of Dermatology, Venereology and Allergology, University Medical Center and Medical Faculty Mannheim, Heidelberg University and Center of Excellence in Dermatology, Mannheim, Germany, [2] European Center for Angioscience, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [3] Next Generation Sequencing Core Facility, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [4] Division of Experimental Therapeutics, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [5] Department of Cardiovascular Physiology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany, [6] Section of Clinical and Molecular Dermatology, Department of Dermatology, Venereology and Allergology, University Medical Center and Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

Endothelial wingless-related integration site (Wnt)-/β-catenin signaling is a key regulator of the tightly sealed blood–brain barrier. In the hepatic vascular niche angiokine-mediated Wnt signaling was recently identified as an important regulator of hepatocyte function, including the determination of final adult liver size, liver regeneration, and metabolic liver zonation. Within the hepatic vasculature, the liver sinusoidal endothelial cells (LSECs) are morphologically unique and functionally specialized microvascular endothelial cells (ECs). Pathological changes of LSECs are involved in chronic liver diseases, hepatocarcinogenesis, and liver metastasis. To comprehensively analyze the effects of endothelial Wnt-/β-catenin signaling in the liver, we used endothelial subtype-specific *Clec4g-iCre* mice to generate hepatic ECs with overexpression of Ctnnb1. In the resultant *Clec4g-iCre*$^{tg/wt}$;*Ctnnb1(Ex3)*$^{fl/wt}$ (*Ctnnb1*$^{OE-EC}$) mice, activation of endothelial Wnt-/β-catenin signaling resulted in sinusoidal transdifferentiation with disturbed endothelial zonation, that is, loss of midzonal LSEC marker lymphatic vessel endothelial hyaluronic acid receptor 1 (Lyve1) and enrichment of continuous EC genes, such as cluster of differentiation *(CD)34* and *Apln*. Notably, gene set enrichment analysis revealed overrepresentation of brain endothelial transcripts. Activation of endothelial Wnt-/β-catenin signaling did not induce liver fibrosis or alter metabolic liver zonation, but *Ctnnb1*$^{OE-EC}$ mice exhibited significantly increased plasma triglyceride concentrations, while liver lipid content was slightly reduced. Ctnnb1 overexpression in arterial ECs of the heart has been reported previously to cause cardiomyopathy. As *Clec4g-iCre* is active in a subset of cardiac ECs, it was not unexpected that *Ctnnb1*$^{OE-EC}$ mice showed reduced

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***Correspondence:**
*Philipp-Sebastian Koch*
*philipp.koch@umm.de*

*†These authors have contributed equally to this work and share first authorship*

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overall survival and cardiac dysfunction. Altogether, balanced endothelial Wnt-/β-catenin signaling in the liver is required for normal LSEC differentiation and for maintenance of normal plasma triglyceride levels.

**Keywords: mice, liver, liver sinusoidal endothelial cells, endothelial cells, triglycerides**

## INTRODUCTION

Liver sinusoidal endothelial cells (LSECs) lining the hepatic sinusoids are a prime example for organ-specific endothelial differentiation. They belong to the group of discontinuous ECs characterized by an incomplete basement membrane and the presence of large fenestrations without diaphragm. LSECs exhibit unique molecular, phenotypic, and functional features and are known to instruct the hepatic vascular niche by cellular interactions and the secretion of paracrine-acting factors called angiokines (Nolan et al., 2013; Augustin and Koh, 2017). For example, LSECs were identified to control liver regeneration by angiocrine wingless-related integration site 2 (Wnt2) and hepatocyte growth factor (Hgf) signaling (Ding et al., 2010; Cao et al., 2017; Zhang et al., 2020). Other highly specialized functions include immunological processes, such as tolerance and defense mechanisms and the clearance of noxious factors from the circulation by a repertoire of scavenger receptors (Schledzewski et al., 2011; Wittlich et al., 2017; Shetty et al., 2018). Interestingly, pathological changes of these highly specialized ECs were shown to contribute to severe liver diseases ranging from steatohepatitis to liver cirrhosis and from hepatocarcinogenesis to liver metastasis (Kostallari and Shah, 2016). During disease processes, LSECs are known to transdifferentiate toward a capillary phenotype revealing a loss of fenestrations and formation of a basement membrane, which is termed "sinusoidal capillarization," thereby aggravating disease progression (Schaffner and Popper, 1963; Lalor et al., 2006). However, the molecular and signaling mechanisms driving sinusoidal capillarization still await detailed analysis.

Notably, recent work by our group could identify the transcription factor GATA-binding factor 4 (GATA4) as a molecular master regulator for LSEC differentiation during liver development and in liver homeostasis. LSEC-restricted deletion of *Gata4* was shown to cause transformation of discontinuous liver sinusoids into continuous capillaries. This sinusoidal transformation in the fetal liver inhibited homing of hematopoietic stem and progenitor cells into the fetal liver resulting in fatal anemia (Geraud et al., 2017), while *Gata4* deletion in the mature vasculature caused hepatopathy and perisinusoidal liver fibrosis (Winkler et al., 2021). A special form of liver sinusoidal capillarization was also demonstrated when endothelial Notch signaling was enhanced resulting in a partial loss of LSEC-specific markers and increased the expression of continuous endothelial cell (CEC) markers; however, lacking the formation of a solid basement membrane or liver fibrogenesis (Wohlfeil et al., 2019).

Another highly conserved transduction pathway known to be involved in several important biological processes, such as liver development, vascular and hepatic differentiation, and tissue homeostasis is the Wnt-/β-catenin signaling pathway (Decaens et al., 2008; Daneman et al., 2009; Wild et al., 2020). In the liver vasculature, Wnt2 was identified as an LSEC-associated molecule with autocrine growth effects (Klein et al., 2008; Geraud et al., 2010), and also as an angiocrine regulator of liver regeneration (Ding et al., 2010). In the meantime, angiocrine Wnt signaling in the liver has become even more important. Hepatic endothelial cells (ECs) not only express Wnt2, they also express Wnt9b, Wnt ligand secretion mediator (Wls), and Wnt potentiator R-Spondin 3 (Geraud et al., 2010; Rocha et al., 2015). These factors are indispensable for the formation of a Wnt-dependent pericentral hepatocyte subpopulation. Loss of this Wnt-signaling machinery results in decreased liver size, perturbations of liver zonation, metabolic maturation, and impaired liver regeneration capacity (Rocha et al., 2015; Wang et al., 2015; Planas-Paz et al., 2016; Leibing et al., 2018; Preziosi et al., 2018).

Considering EC morphogenesis and specification, Wnt signaling emerged as a major contributor in the past decades (Choi et al., 2012). ECs not only express intracellular Wnt molecules and their corresponding Frizzled receptors, but also β-catenin-dependent transcription factors (Masckauchan et al., 2005; Deb, 2014). Hereby, it was shown that β-catenin induces arterialization and loss of venous fate of the embryonic vasculature during development (Duarte et al., 2004; Corada et al., 2010). Moreover, in vascular beds of the central nervous system Wnt-signaling is a key regulator of the integrity of the highly sealed blood–brain barrier (BBB) by controlling the formation of tight junction (TJ) molecules and solute transporters (Liebner et al., 2008; Zhou et al., 2014; Tran et al., 2016; Profaci et al., 2020). LSECs, on the other hand, do not typically express TJs, since permeability and cell trafficking are facilitated by open fenestrations (Geraud et al., 2012). Constitutive activation of β-catenin in the highly permeable ECs of the circumventricular organs in the brain resulted in the expression of BBB markers and downregulation of non-BBB vasculature markers (Benz et al., 2019; Wang et al., 2019). As our study and other previous studies have shown that Wnt2 is a LSEC-specific growth and differentiation factor required for liver regeneration and that autocrine Wnt/β-catenin signaling cross-stimulates the angiogenetic vascular endothelial growth factor receptor 2 pathway, we hypothesized that unbalanced canonical endothelial Wnt signaling in the liver might also impair LSEC differentiation and LSEC-mediated liver function. To test this hypothesis, we generated a novel mouse line with constitutive β-catenin overactivation in LSECs by crossing *Ctnnb1-Ex3*$^{fl/wt}$ with EC subtype-specific *Clec4g-iCre*$^{tg/wt}$ mice (Wohlfeil et al., 2019).

# MATERIALS AND METHODS

## Animals

To generate endothelial subtype-specific Ctnnb1 gain-of-function (GOF) mice (*Ctnnb1*$^{OE-EC}$), *Clec4g-iCre*$^{tg/wt}$ (*Tg(Clec4g-icre)1.1Sgoe* (Wohlfeil et al., 2019) were crossed with *Ctnnb1(Ex3)*$^{fl/wt}$ (*Ctnnb1*$^{tm1Mmt}$) (Harada et al., 1999) mice. Specificity of Cre-activity was analyzed in crosses of *Clec4g-iCre*$^{tg/wt}$ transgenic mice with *R26YFP* (*B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J*) [JAX 006148] (Srinivas et al., 2001) reporter animals. All animals were housed under specific pathogen-free conditions in an animal facility (Heidelberg University). Animal experiments were performed in accordance with Federal Animal Regulations and were institutionally approved by the district government Karlsruhe and performed under institutional guidelines. Mice were sacrificed by cervical dislocation. Liver, heart, kidney, lung, spleen, brain, and intestine weights were measured, and tissue samples were either embedded in the optimum cutting temperature compound (Sakura, Alphen aan den Rijn, The Netherlands) and frozen in liquid nitrogen or fixed in 4% paraformaldehyde at 4°C.

## Isolation of Primary Murine LSECs

Livers, pooled from two mice, were perfused *in situ* via the portal vein with a 0.05% collagenase containing amino acid/saccharide calcium-deprived medium (C2674, Sigma–Aldrich, Taufkirchen, Germany), dissected, mechanically minced, digested at 38°C in a collagenase/Gey's balanced salt solution (G9779, Sigma–Aldrich) and filtered through a 250 μm mesh. Cells were separated by a 35% Nycodenz (1002424, Axis-Shield, Alere Technologies, Oslo, Norway) gradient. Next, LSECs were isolated by magnetic-activated cell sorting using anti-CD146 MicroBeads (ME-9F1, 130-092-007, Miltenyi Biotech, Bergisch Gladbach, Germany) according to the instructions of the manufacturers.

## Quantitative Reverse-Transcription PCR

RNA was extracted from primary ECs using EZNA Total-RNA-Kit I (OMEGA Biotec, Norcross, GA, United States). Complementary DNA (cDNA) was synthesized with RevertAid H-Minus M-MuLV Reverse Transcriptase (ThermoScientific, Waltham, MA, United States). Quantitative PCR was performed on a qTOWER 3 G touch thermal cycler (Analytik Jena) using innuMIX qPCR SyGreen Sensitive (845-AS-1310200, Analytik Jena, Jena, Germany). Normalized expression values were calculated using the Pfaffl method considering amplification efficiency values determined by standard curves (Pfaffl, 2001).

## RNA *in situ* Hybridization

Liver tissue was sectioned at 4 μm. RNA *in situ* hybridization (ISH) was conducted using RNAscope 2.5 HD Red (322350, Advanced Cell Diagnostics, Newark, CA, United States) kits with mouse-specific probes against the positive control mouse *Ppib* (*Cyclophilin B*) gene, *Mus musculus* (Mm)-*Bmp2*-E3-Channel 1 (1545–2347 NM_007553.3), Mm-*Hgf*-Channel 1 (1120–2030 NM_010427.4), Mm-*Wnt2*-Channel 1 (857–2086 NM_023653.5), Mm-*Wnt9b*-Channel 1 (706–1637 NM_011719.4), Mm-*Stab1*-Channel 1 (488-1320 NM_138672.2), and Mm-*Stab2*-Channel 1 (4249–5075 NM_138673.2) according to the protocols of the manufacturer.

## Histology and Immunofluorescence

Tissue samples fixed by 4% paraformaldehyde at room temperature for 48–72 h, were subsequently transferred into phosphate-buffered saline (PBS), dehydrated in a graded alcohol series, and embedded in paraffin. Paraffin-embedded tissues were sectioned in 4 μm. For hematoxylin & eosin (H&E), periodic acid–Schiff (PAS), Oil Red O (ORO), Prussian blue, and Sirius red staining, samples were processed according to the standard protocols provided by the manufacturer. For immunofluorescence (IF), cryosections (7 μm) were air-dried, fixed in 4% paraformaldehyde (PFA) or acetone, rehydrated in PBS (A0964.9050, VWR International, Radnor, PA, United States) and blocked in 5% donkey serum (017-000-121, Dianova, Hamburg, Germany) in PBS for 30 min. Primary antibodies were incubated overnight at 4°C. Sections were washed three times in PBS before incubation with appropriate Alexa Fluor–coupled secondary antibodies for 45 min at room temperature. Paraffin-embedded sections were baked at 60°C overnight, after which they were deparaffinized with xylol and rehydrated using ethanol in decreasing concentrations. Antigen retrieval of tissue sections was carried out with epitope retrieval solution (Zytomed Systems, Berlin, Germany) at either pH 6, 8, or 9. Primary antibody was incubated for 2 h at room temperature or overnight at 4°C. Sections were washed three times in PBS before incubation of appropriate secondary antibodies for 1 h at room temperature. Nuclei were counterstained with 4′,6-diamidin-2-phenylindol (DAPI) (D1306, Thermo Fisher Scientific, Waltham, MA, United States). Finally, sections were thoroughly washed in PBS before mounting with Dako fluorescent mounting medium (Dako, Agilent technologies, Santa Clara, CA, United States). Sections were photographed with ECLIPSE Ci microscope (Nikon, Alzenau, Bavaria, Germany) or ECLIPSE Ni-E microscope (Nikon). Immunofluorescence images were acquired in a sequential mode as a series of *z*-axis images and processed with NIS-Elements AR 5.02 (Nikon Instruments, Tokyo, Japan) and ImageJ 1.52e software (NIH, Bethesda, MD, United States). Using NIS-Elements AR 5.02, images were background corrected (rolling ball 7.5 pixels), deconvoluted, and focused to one plane.

For the quantification of IF images, three representative areas per sample were chosen. For each image, binary masks of the representative channels were created using automated threshold functions (Otsu, MaxEntropy) in ImageJ. The resulting binary masks were quantified for the area, or the number of particles (10-infinite pixels) using ImageJ functions "Measure" and "Analyze Particles." The "Mean gray value" represents the sum of the gray values of all the pixels within the selected images divided by the number of all pixels. For Ki-67 quantification, only Ki-67 staining was included that overlaid with DAPI-positive nucleus staining to exclude unspecific signal. To this end, we used the "Image Calculator" in ImageJ with "AND" as operator for Ki-67 and DAPI channels. For quantification of RNA ISH or ORO images, three representative areas per sample were chosen. RGB images were split into separate

channels corresponding to three determined colors by using the "Color deconvolution" command in ImageJ. The images displaying the region of interest were further processed by setting color thresholds. Finally, the area of particles (>30 pixels) was measured, analyzed, and calculated in percentage (%) of the whole image area.

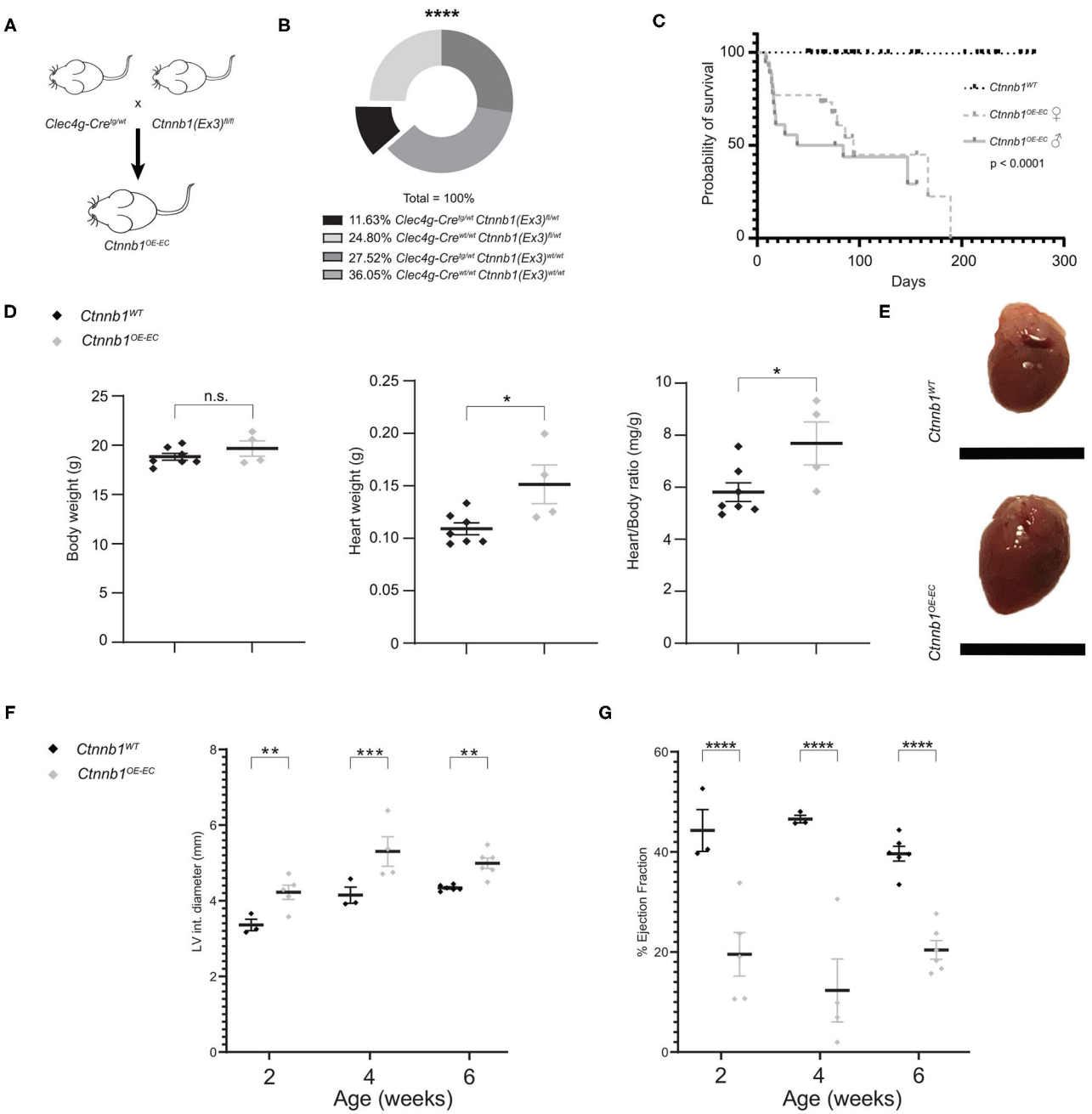


**FIGURE 1 |** *Ctnnb1*$^{OE-EC}$ mice have a low survival probability and suffer from cardiac dysfunction **(A)** *Ctnnb1*$^{OE-EC}$ mice [*Clec4g-iCre*$^{tg/wt}$;*Ctnnb1(Ex3)*$^{fl/wt}$] were generated by crossing *Clec4g-iCre*$^{tg/wt}$ with *Ctnnb1(Ex3)*$^{fl/fl}$ mice. **(B)** Mendelian frequency of *Clec4g-iCre*$^{tg/wt}$;*Ctnnb1(Ex3)*$^{fl/wt}$ mice. ****$p < 0.0001$. **(C)** Kaplan–Meier survival curves for control (*Ctnnb1*$^{WT}$) and *Ctnnb1*$^{OE-EC}$ mice. The probability of survival is shown for *Ctnnb1*$^{OE-EC}$ female ($n = 26$) vs. *Ctnnb1*$^{OE-SEC}$ male ($n = 18$) vs. *Ctnnb1*$^{WT}$ mice ($n > 65$). **(D)** Body weight, heart weight, heart-to-body weight ratio of 2- to 3-month-old *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice (female, $n \geq 4$). Results are represented as mean ± SEM. ns, not significant; *$p < 0.05$. **(E)** Macroscopic heart images of 3-month-old *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice (female, $n = 4$). Scale bar 1 cm. **(F)** Left ventricle (LV) interior diameter and **(G)** ejection fraction as determined by echocardiography of 2-, 4-, and 6-week-old *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n \geq 3$). Results are represented as mean ± SEM. **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

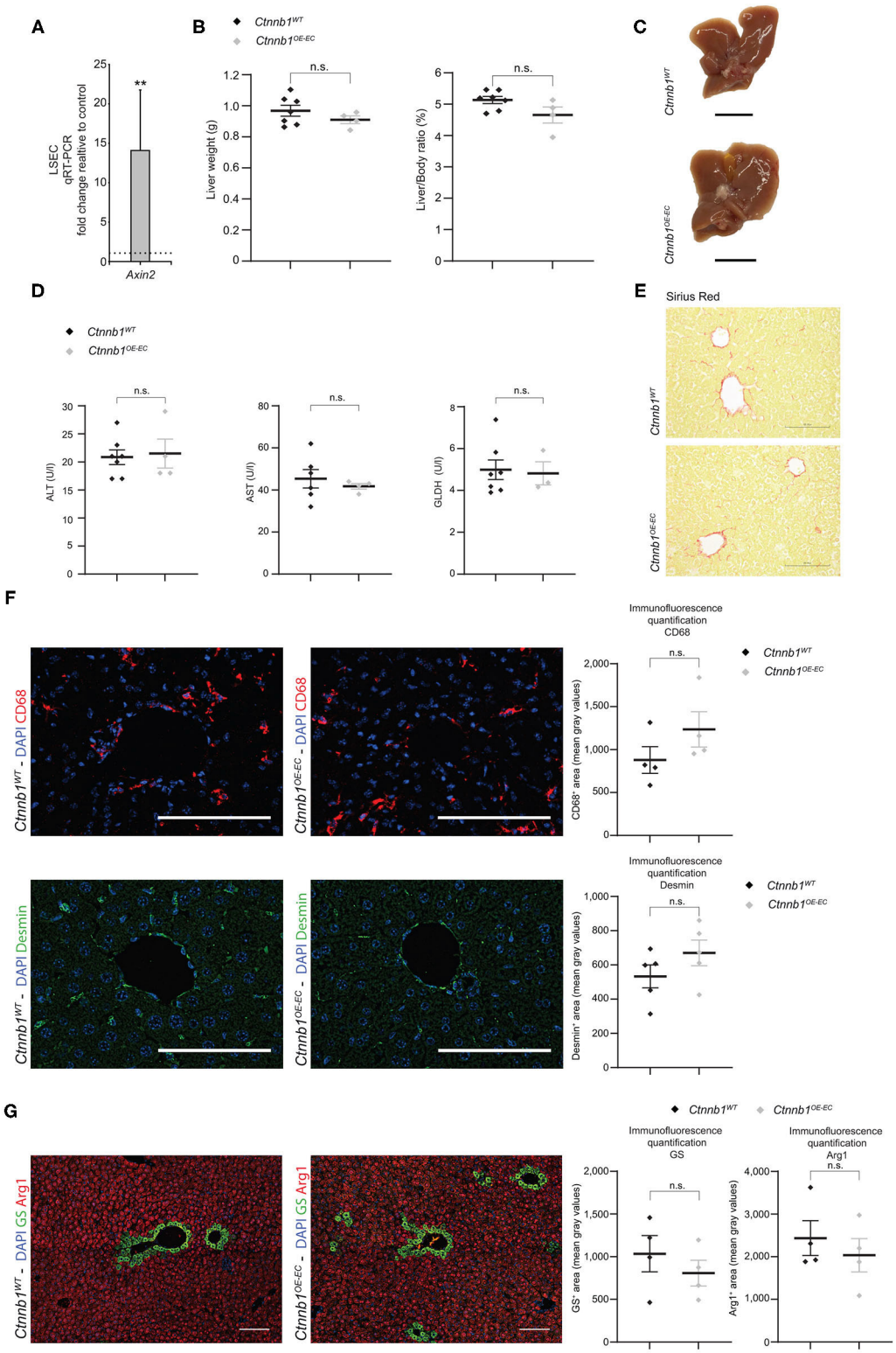


**FIGURE 2 |** Hepatic endothelial *Ctnnb1* overactivation does not lead to hepatopathy and fibrosis. **(A)** qRT-PCR for axis inhibition protein 2 (*Axin2*) of cDNA from freshly isolated LSECs of 2-months-old $Ctnnb1^{OE-EC}$ mice compared to corresponding $Ctnnb1^{WT}$ controls ($n = 3$). β-Actin was used as housekeeping gene. $^{**}p <$ 0.01. **(B)** Liver weight, liver-to-body weight ratio of 2- to 3-month-old $Ctnnb1^{WT}$ and $Ctnnb1^{OE-EC}$ mice (female, $n \geq 4$). Results are represented as mean ± SEM. ns, *(Continued)*

**FIGURE 2 |** not significant. **(C)** Macroscopic liver images of 3-month-old *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice (female, $n = 4$). Scale bar 1 cm. **(D)** Liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and glutamate dehydrogenase (GLDH)] in serum of 2- to 3-month-old female *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n \geq 3$). Results are represented as mean ± SEM. n.s., not significant. **(E)** Sirius red staining of liver sections of 2- to 3-month-old male *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n = 4$). Scale bar 100 μm. **(F)** Immunofluorescence (IF) staining of DAPI, CD68 and Desmin, and CD68 and Desmin quantification in the liver of 2- to 3-month-old female *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n \geq 4$). Scale bar 100 μm. Results are represented as mean ± SEM. ns, not significant. **(G)** IF staining of DAPI, glutamine synthetase (GS) and arginase (Arg1), and GS and Arg1 quantification in the liver of 2- to 3-month-old *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n = 4$). Scale bar 100 μm. Results are represented as mean ± SEM. ns, not significant.

## Antibodies

Primary antibodies: rat anti-Endomucin (14-5851-82, eBioscience, San Diego, CA, United States), goat anti-Lyve1 (AF2125, R&D Systems, Minneapolis, MN, United States), rat anti-mouse/human GATA-4 (14-9980-82, Thermo Fisher Scientific), rat anti-mouse CD68 (137002, BioLegend, San Diego, CA, United States), rabbit anti-Desmin (ab15200, Abcam, Cambridge, Cambs., UK), rabbit anti-glutamine synthetase (G2781, Sigma–Aldrich, Taufkirchen, Bavaria, Germany), goat anti-arginase I (sc-18351, Santa Cruz Biotechnology, Dallas, TX, United States), rat anti-Ki67 (14-5698-82, eBioscience), polyclonal rabbit anti- green fluorescent protein/yellow fluorescent protein (YFP) (A11122, Molecular Probes, Eugene, OR, United States), rat anti-CD31 (102502, BioLegend), SMA-antibody (ab5694, Abcam), goat anti-CD32b (AF1460, R&D Systems), rabbit anti-Collagen type I (R1038, Acris, Hiddenhausen, North Rhine-Westphalia, Germany), rabbit anti-Collagen type III alpha 1 chain (R1040, Acris), rabbit anti-Collagen IV (GTX19808, Genetex, Irvine, CA, United States), rabbit anti-Cyp2E1 (HPA009128, Sigma–Aldrich), rabbit anti-Claudin 5 (34-1600, Thermo Fisher Scientific), goat anti-Podocalyxin (AF1556, R&D Systems), rabbit anti-Cav1 (N-20, Santa Cruz Biotechnology), rabbit anti- intracellular adhesion molecule 1 (ICAM1) (10020-1-AP, Proteintech, Rosemont, IL, United States), goat anti-mouse vascular endothelial (VE)-cadherin (AF1002, R&D Systems), goat anti- vascular cell adhesion molecule (VCAM)-1/CD106 (AF643, R&D Systems). Secondary antibodies: Alexa-Fluor 488, Alexa-Fluor 647, and cyanine 3-conjugated secondary antibodies were purchased from Dianova (Hamburg, Germany).

## Microarray Processing and Statistical Analysis

Gene expression profiling was performed using arrays MoGene-2_0-st from Affymetrix (Santa Clara, CA, United States). Biotinylated antisense cDNA and arrays hybridization were performed according to the recommendations of the manufacturer using the GeneChip WT Plus Reagent Kit and the GeneChip Hybridization, Wash and Stain Kit (both from Thermo Fisher Scientific). A Custom CDF Version 22 with ENTREZ-based gene definitions was used to annotate the arrays. The raw fluorescence intensity was robust multiarray analysis background corrected and values were normalized applying quantile normalization. Differential gene expression was analyzed with the one-way-ANOVA, using a commercial software package SAS JMP15 Genomics, version10, from SAS (SAS Institute, Cary, NC, United States). A false-positive rate of $a = 0.05$ with FDR correction was taken as the level of significance.

To determine whether defined lists (or sets) of genes exhibit a statistically significant bias in their distribution, we performed a gene set enrichment analysis (GSEA). GSEA (Subramanian et al., 2005) was carried out using R 3.6.1. clusterProfiler 3.12.0 (Yu et al., 2012), fgsea 1.10.0 (Korotkevich et al., 2021), the molecular signatures database (MSigDB) v6.2 hallmark gene set collection (Liberzon et al., 2015), and self-defined gene lists were used. Gene lists for LSECs and CECs were used as previously described (Winkler et al., 2021). The gene set for brain ECs was defined using published single-cell RNA seq data (Sabbagh et al., 2018). Inclusion criteria were fold change ≥ 2 for brain *vs.* liver ECs and at least 10 transcripts per million in liver ECs to exclude less-expressed genes. Overrepresentation analysis (ORA) of Gene Ontology terms was performed with the enrichR (Chen et al., 2013) package in R 3.6.1 for all significantly regulated genes. Heatmaps were created with the ComplexHeatmap package (Gu et al., 2016).

The raw and normalized gene expression profiling data have been deposited in the NCBI Gene Expression Omnibus and are accessible through GEO Series accession number GSE175777 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175777).

## Blood Parameters

Serum was analyzed for the following routine parameters: alanine aminotransferase (ALA), aspartate aminotransferase (AST), and glutamate dehydrogenase (GLDH), cholesterol, triglycerides, glucose, and total protein (Roche cobas c 311 analyser, Roche Diagnostics, Basel, Switzerland).

## Hepatic Triglycerides

Snap frozen liver tissue (100 mg) was homogenized in 5% NP-40 solution (74385, Merck) and heated for 5 min in a shaking dry incubator (ThermoMixer C, Eppendorf, Hamburg, Germany) at 80–100°C. After cooling to room temperature, the heating was repeated in order to solubilize all triglycerides. After centrifugation for 2 min at top speed (Centrifuge 5417 R, Eppendorf) the supernatant was diluted 10-fold in distilled water and used to determine the triglyceride content based on the protocol of the Triglyceride Quantification Colorimetric/Fluorometric Kit manufacturer (K622, BioVision, Mountain View, CA, United States).

## Transthoracic Echocardiography

For echocardiography, mice were anesthetized with 0.5–1.0% isoflurane and placed on a heating pad to maintain body temperature. Non-invasive, echocardiographic parameters were recorded with a linear 50 MHz transducer (Vevo 3100 system

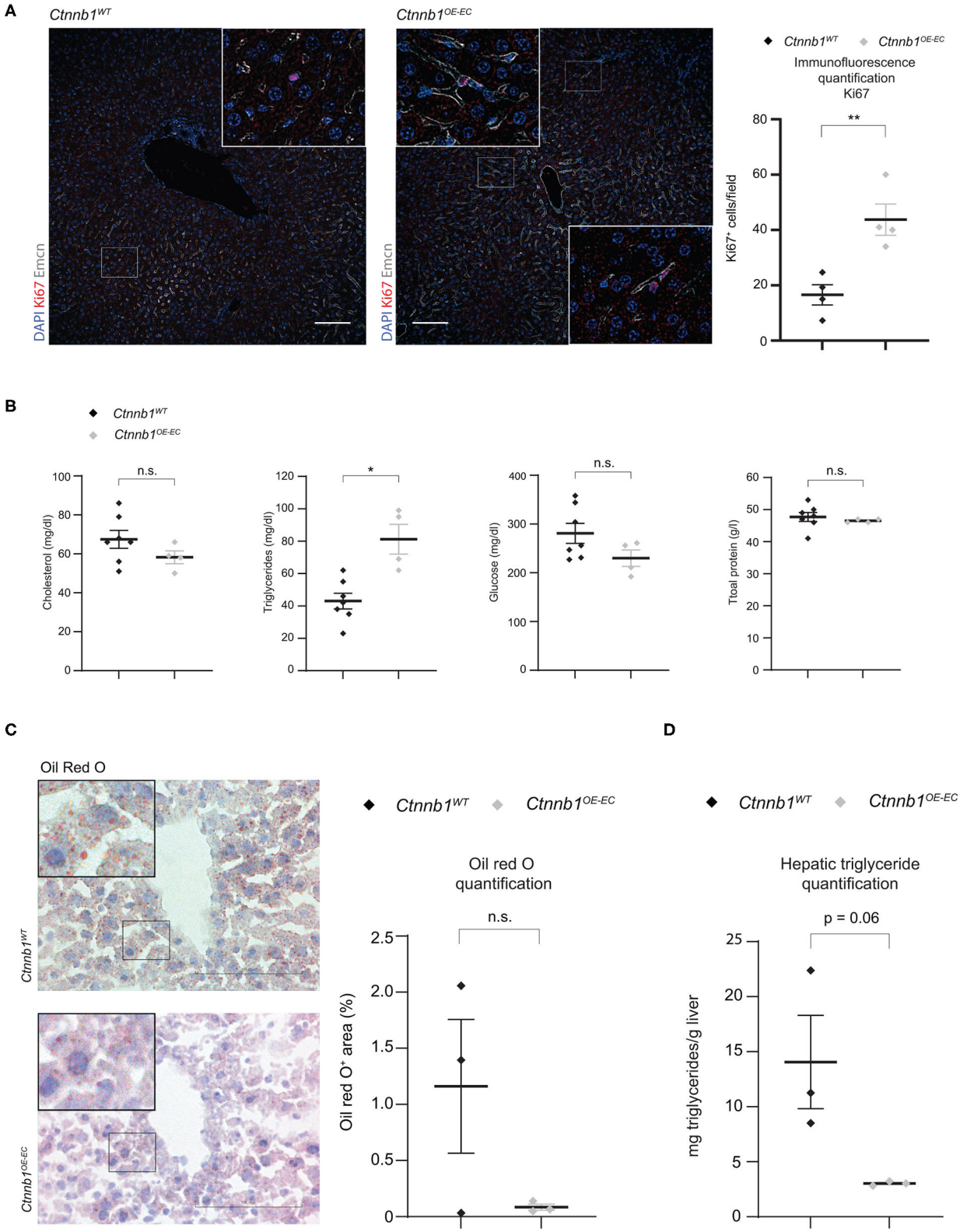


**FIGURE 3 |** *Ctnnb1*$^{OE-EC}$ mice display enhanced hepatic endothelial cell proliferation, serum hypertriglyceridemia, and decreased lipid accumulation in the liver. **(A)** IF staining of DAPI, Ki67, and Emcn, and Ki67 quantification in the liver of 2- to 3-month-old female *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n = 3$). Scale bar 100 μm. Results are represented as mean ± SEM. $^{**}p < 0.01$. **(B)** Serum levels of cholesterol, triglycerides, and glucose in 2- to 3-month-old *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$

*(Continued)*

**FIGURE 3 |** mice (female, $n \geq 5$). Results are represented as mean ± SEM. $^{**}p < 0.01$. **(C)** Oil Red O (ORO) staining and quantification of livers of 3-month-old female *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n = 3$). Scale bar 100 μm. Results are represented as mean ± SEM. ns, not significant. **(D)** Hepatic triglyceride concentration of murine liver tissue of 3-month-old female *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n = 3$). $^{*}p < 0.05$.

with MX700 transducer, Visualsonics, Toronto, Canada) in parasternal long-axis B- & M-mode, and measured post-processing, which comprised heart rate, left ventricle (LV) posterior and anterior wall thickness, and LV internal diameter at both end-systole and end-diastole. From that, LV volume, LV ejection fraction, and cardiac output were calculated with the Vevo Workstation 5.5.0 and the integrated cardiac measurement package.

## Statistics

Statistical analysis was performed with SigmaPlot 11 Software (Systat Software GmbH, Germany). For pairwise comparisons, the *t*-test was used when normality was proved. Differences between data sets with $p < 0.05$ were considered statistically significant. Data are presented as means with error bars indicating standard error.

# RESULTS

## Generation and Characterization of Adult β-Catenin-Overactivated HEC Mice

EC subtype-specific *Clec4g-iCre* mice (Wohlfeil et al., 2019) were used to generate mice with Ctnnb1 GOF in LSECs (**Figure 1A**). *Ctnnb1*$^{OE-EC}$ (*Clec4g-iCre*$^{tg/wt}$;*Ctnnb1(Ex3)*$^{fl/wt}$) mice were viable but were born at a lower Mendelian frequency than expected (**Figure 1B**) and suffered from a reduced overall survival rate (**Figure 1C**). While bodyweight was not altered, heart weight as well as heart weight/body weight ratio were significantly increased in *Ctnnb1*$^{OE-EC}$ mice (**Figures 1D,E**). As Cre-activity was previously described in ECs of the heart in *Clec4g-iCre* mice (Wohlfeil et al., 2019), a comprehensive analysis of *Clec4g-iCre;R26YFP* reporter mice was performed for this organ. Reporter activity was present in CD31$^{+}$ ECs of the heart (**Supplementary Figure 1A**). Specifically, YFP positivity was observed in the endocardium, including endomucin (Emcn)$^{+}$ endocardial trabeculae (Rhee et al., 2018) as well as in CD31$^{+}$ αSMA$^{+}$ coronary veins and arteries (Zhang et al., 2005) (**Supplementary Figures 1A,B**). In contrast, LYVE1$^{+}$ lymphatic vessels were YFP negative (**Supplementary Figure 1B**). In *Ctnnb1*$^{OE-EC}$ mice, echocardiography revealed progressive cardiac dysfunction, which is comparable to the phenotype obtained after β-catenin GOF mutation in arterial ECs of the heart by using *Bmx-CreER*$^{T2}$ transgenic mice (Nakagawa et al., 2016). *Ctnnb1*$^{OE-EC}$ mice displayed increased end-diastolic left ventricle internal diameters and volumes (**Figure 1F**; **Supplementary Figure 2A**) and a reduction in wall thickness of the left ventricle (**Supplementary Figures 2B,C**). The ejection fraction was significantly reduced starting with 2 weeks of age (**Figure 1G**), whereas the cardiac output was first reduced starting with 4 weeks of age (**Supplementary Figure 2D**). A routine histochemical staining of internal organs such as the kidneys, lungs, spleen, brain, and intestine were gross morphologically unremarkable (**Supplementary Figure 3**).

Endothelial β-catenin overactivation in the liver was confirmed by quantitative reverse-transcription PCR (qRT-PCR), which showed significantly elevated expression of Wnt-/β-catenin downstream target gene axis inhibition protein 2 (*Axin2*) in isolated LSECs from *Ctnnb1*$^{OE-EC}$ mice (**Figure 2A**). Liver size, liver weight, and liver/body weight ratio were not significantly altered in *Ctnnb1*$^{OE-EC}$ mice (**Figures 2B,C**). Basic liver function tests did not show elevated levels of ALT, AST, and GLDH (**Figure 2D**). Upon Sirius red staining, no signs of fibrosis were present in the *Ctnnb1*$^{OE-EC}$ livers (**Figure 2E**). In line with the absence of collagen deposition upon Sirius red staining, no changes in perisinusoidal collagen I, III, or basement membrane collagen IV deposition were seen (**Supplementary Figure 4A**). Additionally, no obvious alterations were seen in livers of *Ctnnb1*$^{OE-EC}$ mice upon H&E histology, PAS, and Prussian blue staining (**Supplementary Figure 4B**). Likewise, Kupffer cells or hepatic stellate cells (HSC) were not altered in quantity, as analyzed by IF for CD68 or Desmin, respectively (**Figure 2F**). Co-IF of marker proteins for metabolic liver zonation revealed no changes in zonated expression of Glul/GS and Cyp2E1 in pericentral or Arg1 in periportal and midlobular hepatocytes (**Figure 2G**; **Supplementary Figure 4C**). Notably, there was a significant increase in the Ki67-positivity in ECs from *Ctnnb1*$^{OE-EC}$ livers, while the proliferation of hepatocytes did not show changes (**Figure 3A**).

Recently, we could demonstrate that EC-derived Wnt signaling controls metabolic liver zonation and alters lipid metabolism (Leibing et al., 2018). Although metabolic liver zonation was not affected by β-catenin GOF mutation, we performed a comprehensive metabolic screening including serum parameters such as total protein, cholesterol, triglycerides, and glucose (**Figure 3B**). Interestingly, *Ctnnb1*$^{OE-EC}$ mice showed significantly increased serum levels of triglycerides (**Figure 3B**). Liver tissue of *Ctnnb1*$^{OE-EC}$ mice showed slightly reduced lipid storage upon Oil Red O staining (**Figure 3C**) and a tendency of decreased levels of hepatic triglycerides as measured by a colorimetric assay (**Figure 3D**).

## Hepatic Endothelial β-Catenin GOF Mutation Causes Molecular Transdifferentiation of LSECs

To identify β-catenin-dependent molecular alterations in LSECs, we performed comprehensive Affymetrix DNA microarray gene expression profiling of isolated primary LSECs from *Ctnnb1*$^{WT}$ control and *Ctnnb1*$^{OE-EC}$ animals. β-catenin GOF mutation in LSECs resulted in the significant dysregulation of 128 genes as compared to control LSECs (**Table 1**). GSEA of LSECs isolated from *Ctnnb1*$^{WT}$ control and *Ctnnb1*$^{OE-EC}$ animals revealed significant pathway alterations

**TABLE 1 |** Differentially expressed genes (DEGs) in *Ctnnb1*$^{OE-EC}$-LSECs compared to wild-type controls.

| Gene symbol | Gene title | Fold change *Ctnnb1*$^{OE-EC}$ > *Ctnnb1*$^{WT}$ | Adjusted p-value for Diff of genotype = *Ctnnb1*$^{OE-EC}$–*Ctnnb1*$^{WT}$ |
|---|---|---|---|
| *Slc35f2* | Solute carrier family 35, member F2 | 35.13809 | 0.000397 |
| *Apln* | Apelin | 10.30015045 | 0.016367 |
| *Susd4* | Sushi domain containing 4 | 9.257366289 | 0.007219 |
| *Csf2rb2* | Colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage) | 8.781086232 | 0.041067 |
| *Cd34* | CD34 antigen | 8.598926088 | 0.001273 |
| *Selp* | Selectin, platelet | 8.373297581 | 0.037503 |
| *Lypd6* | LY6/PLAUR domain containing 6 | 8.152209698 | 0.004191 |
| *Glp1r* | Glucagon-like peptide 1 receptor | 7.081854471 | 0.041067 |
| *Myo1b* | Myosin IB | 6.942870328 | 0.023473 |
| *St8sia2* | ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2 | 5.709992179 | 0.022697 |
| *Hunk* | Hormonally upregulated Neu-associated kinase | 5.184396379 | 0.045899 |
| *St8sia4* | ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 | 4.998737335 | 0.0049 |
| *Atp10a* | ATPase, class V, type 10A | 4.856467229 | 0.012795 |
| *Tcf7* | Transcription factor 7, T cell specific | 4.800597937 | 0.013887 |
| *Mal* | Myelin and lymphocyte protein, T cell differentiation protein | 4.715053595 | 0.025665 |
| *Ptgis* | Prostaglandin I2 (prostacyclin) synthase | 4.52969164 | 0.030765 |
| *Fkbp10* | FK506 binding protein 10 | 4.43037992 | 0.039368 |
| *Axin2* | Axin 2 | 4.416998695 | 0.022697 |
| *Pla2g16* | Phospholipase A2, group XVI | 4.319974957 | 0.039368 |
| *Il17ra* | Interleukin 17 receptor A | 3.86500417 | 0.031256 |
| *Disp1* | Dispatched RND transporter family member 1 | 3.826121183 | 0.031256 |
| *Ptgfrn* | Prostaglandin F2 receptor negative regulator | 3.663983702 | 0.023473 |
| *Greb1l* | Growth regulation by estrogen in breast cancer-like | 3.513247405 | 0.0049 |
| *Sptb* | Spectrin beta, erythrocytic | 3.406728181 | 0.016367 |
| *Aqp11* | Aquaporin 11 | 3.384139553 | 0.026891 |
| *Extl3* | Exostoses (multiple)-like 3 | 3.381491933 | 0.037503 |
| *Cttnbp2* | Cortactin binding protein 2 | 3.165960888 | 0.022697 |
| *Kif21b* | Kinesin family member 21B | 3.103400943 | 0.002999 |
| *Fam213a* | Family with sequence similarity 213, member A | 3.092980639 | 0.024278 |
| *Auts2* | Autism susceptibility candidate 2 | 3.014691756 | 0.046838 |
| *Tspan6* | Tetraspanin 6 | 2.872077594 | 0.046282 |
| *Lrig1* | Leucine-rich repeats and immunoglobulin-like domains 1 | 2.738931683 | 0.046282 |
| *Pi16* | Peptidase inhibitor 16 | 2.427155192 | 0.037503 |
| *Laptm4b* | Lysosomal-associated protein transmembrane 4B | 2.318212303 | 0.011483 |
| *Slc7a6* | Solute carrier family 7 (cationic amino acid transporter, y+ system), member 6 | 2.267050692 | 0.024407 |
| *Ptprg* | Protein tyrosine phosphatase, receptor type, G | 2.158891183 | 0.022697 |
| *Cachd1* | Cache domain containing 1 | 2.065569075 | 0.007219 |
| *Rasgrp4* | RAS guanyl releasing protein 4 | 2.057762756 | 0.045899 |
| *Klhl29* | Kelch-like 29 | 2.027131525 | 0.036831 |
| *Bambi* | BMP and activin membrane-bound inhibitor | 2.016141198 | 0.044925 |
| *Mlec* | Malectin | 2.003811198 | 0.022697 |
| *Ppp1r9a* | Protein phosphatase 1, regulatory (inhibitor) subunit 9A | 1.948922878 | 0.036956 |
| *1810058I24Rik* | RIKEN cDNA 1810058I24 gene | 1.899752884 | 0.038628 |
| *Osbp2* | Oxysterol binding protein 2 | 1.86993046 | 0.024407 |
| *Gm13889* | Predicted gene 13889 | 1.808734268 | 0.036956 |
| *Vim* | Vimentin | 1.808581233 | 0.041067 |
| *Gnai1* | Guanine nucleotide binding protein (G protein), alpha inhibiting 1 | 1.795430532 | 0.036831 |
| *Slc1a4* | Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 | 1.731445442 | 0.042161 |

*(Continued)*

**TABLE 1 |** Continued

| Gene symbol | Gene title | Fold change $Ctnnb1^{OE-EC}$ > $Ctnnb1^{WT}$ | Adjusted p-value for Diff of genotype = $Ctnnb1^{OE-EC}$–$Ctnnb1^{WT}$ |
|---|---|---|---|
| *Cdc14a* | CDC14 cell division cycle 14A | 1.727805031 | 0.041067 |
| *Fbl* | Fibrillarin | 1.67657139 | 0.045899 |
| *Lmo2* | LIM domain only 2 | 1.650427031 | 0.043595 |
| *1110051M20Rik* | RIKEN cDNA 1110051M20 gene | 1.601538653 | 0.036831 |
| *Cpt1c* | Carnitine palmitoyltransferase 1c | 1.577681949 | 0.042456 |
| *Fxyd5* | FXYD domain-containing ion transport regulator 5 | 1.552882564 | 0.038628 |
| *Lrrc75a* | Leucine rich repeat containing 75A | 1.496322239 | 0.046203 |
| *Pgap1* | Post-GPI attachment to proteins 1 | 1.490320435 | 0.046282 |
| *Zfp36l1* | Zinc finger protein 36, C3H type-like 1 | 1.474391469 | 0.037651 |
| *Mir3092* | microRNA 3092 | 1.440648261 | 0.03784 |
| *Ppic* | Peptidylprolyl isomerase C | 1.435613585 | 0.043595 |
| *Ppdpf* | Pancreatic progenitor cell differentiation and proliferation factor | 1.336327784 | 0.041067 |
| *Cdk4* | Cyclin-dependent kinase 4 | 1.32815528 | 0.037503 |
| *St3gal4* | ST3 beta-galactoside alpha-2,3-sialyltransferase 4 | 1.281026042 | 0.036831 |
| *D630024D03Rik* | RIKEN cDNA D630024D03 gene | 1.238041514 | 0.037503 |
| *Cdc45* | Cell division cycle 45 | 1.230144783 | 0.022697 |
| *Eif4g1* | Eukaryotic translation initiation factor 4, gamma 1 | 1.143713408 | 0.043595 |
| *Atp6v1d* | ATPase, H+ transporting, lysosomal V1 subunit D | 0.893603982 | 0.048858 |
| *Tmx3* | Thioredoxin-related transmembrane protein 3 | 0.84721687 | 0.048858 |
| *Olfr1033* | Olfactory receptor 1033 | 0.83993199 | 0.042103 |
| *Ergic2* | ERGIC and golgi 2 | 0.834146565 | 0.024407 |
| *Aqp1* | Aquaporin 1 | 0.828501756 | 0.046731 |
| *Glra2* | Glycine receptor, alpha 2 subunit | 0.817240036 | 0.031256 |
| *Gm26744* | Predicted gene, 26744 | 0.810903036 | 0.046282 |
| *Spag9* | Sperm associated antigen 9 | 0.802558367 | 0.036831 |
| *Rnf115* | Ring finger protein 115 | 0.80194744 | 0.036831 |
| *Cd47* | CD47 antigen (Rh-related antigen, integrin-associated signal transducer) | 0.800896378 | 0.031314 |
| *Dpp4* | Dipeptidylpeptidase 4 | 0.794955116 | 0.041067 |
| *Crebl2* | cAMP responsive element binding protein-like 2 | 0.788857661 | 0.022697 |
| *Atp6ap2* | ATPase, H+ transporting, lysosomal accessory protein 2 | 0.782409663 | 0.024407 |
| *Zfp763* | Zinc finger protein 763 | 0.779090085 | 0.007745 |
| *Fez2* | Fasciculation and elongation protein zeta 2 (zygin II) | 0.762992727 | 0.036831 |
| *Zfp715* | Zinc finger protein 715 | 0.744750505 | 0.048858 |
| *Cyb561d1* | Cytochrome b-561 domain containing 1 | 0.744262296 | 0.048858 |
| *Extl2* | Exostoses (multiple)-like 2 | 0.728699546 | 0.048068 |
| *Golga7* | Golgi autoantigen, golgin subfamily a, 7 | 0.723018512 | 0.026891 |
| *Tgoln1* | Trans-golgi network protein | 0.721857092 | 0.036831 |
| *Tpm3* | Tropomyosin 3, gamma | 0.717139012 | 0.044415 |
| *Ggh* | Gamma-glutamyl hydrolase | 0.714504325 | 0.048882 |
| *Scrn3* | Secernin 3 | 0.707136697 | 0.046282 |
| *Irak2* | Interleukin-1 receptor-associated kinase 2 | 0.68925238 | 0.037503 |
| *Tmem170b* | Transmembrane protein 170B | 0.685022667 | 0.046847 |
| *Dgke* | Diacylglycerol kinase, epsilon | 0.681193453 | 0.024407 |
| *Itga1* | Integrin alpha 1 | 0.676125877 | 0.041067 |
| *Sdccag8* | Serologically defined colon cancer antigen 8 | 0.662856645 | 0.041067 |
| *Hspa12a* | Heat shock protein 12A | 0.647460358 | 0.046907 |
| *Nceh1* | Neutral cholesterol ester hydrolase 1 | 0.641787883 | 0.042161 |
| *Impact* | Impact, RWD domain protein | 0.641068766 | 0.022697 |
| *Nlrc3* | NLR family, CARD domain containing 3 | 0.616870496 | 0.024407 |
| *Pitpnm1* | Phosphatidylinositol transfer protein, membrane-associated 1 | 0.607880798 | 0.024407 |

*(Continued)*

**TABLE 1** | Continued

| Gene symbol | Gene title | Fold change $Ctnnb1^{OE-EC}$ > $Ctnnb1^{WT}$ | Adjusted p-value for Diff of genotype = $Ctnnb1^{OE-EC}$–$Ctnnb1^{WT}$ |
|---|---|---|---|
| *Gm19663* | Predicted gene, 19663 | 0.601230962 | 0.035201 |
| *Inpp4b* | Inositol polyphosphate-4-phosphatase, type II | 0.598629428 | 0.048227 |
| *Pde3b* | Phosphodiesterase 3B, cGMP-inhibited | 0.586483967 | 0.036956 |
| *P2ry10b* | Purinergic receptor P2Y, G-protein coupled 10B | 0.58265069 | 0.044415 |
| *Ldhb* | Lactate dehydrogenase B | 0.575509392 | 0.031256 |
| *Smco4* | Single-pass membrane protein with coiled-coil domains 4 | 0.546281346 | 0.039368 |
| *Gm14005* | Predicted gene 14005 | 0.545796227 | 0.046282 |
| *A630072L19Rik* | RIKEN cDNA A630072L19 gene | 0.545449977 | 0.037503 |
| *Cyp7b1* | Cytochrome P450, family 7, subfamily b, polypeptide 1 | 0.537648924 | 0.039821 |
| *Ldb2* | LIM domain binding 2 | 0.526662693 | 0.036831 |
| *Nudt12* | Nudix (nucleoside diphosphate linked moiety X)-type motif 12 | 0.522324687 | 0.037503 |
| *Cfh* | Complement component factor h | 0.521077661 | 0.036831 |
| *Ptpru* | Protein tyrosine phosphatase, receptor type, U | 0.517441974 | 0.042879 |
| *Pgghg* | Protein glucosylgalactosylhydroxylysine glucosidase | 0.479186666 | 0.048882 |
| *Acer3* | Alkaline ceramidase 3 | 0.472763137 | 0.036831 |
| *Ceacam1* | Carcinoembryonic antigen-related cell adhesion molecule 1 | 0.472603157 | 0.041067 |
| *Ccdc88c* | Coiled-coil domain containing 88C | 0.469087339 | 0.027307 |
| *Fam189a2* | Family with sequence similarity 189, member A2 | 0.467888229 | 0.041067 |
| *Cysltr2* | Cysteinyl leukotriene receptor 2 | 0.442737637 | 0.023473 |
| *Gramd1c* | GRAM domain containing 1C | 0.42091094 | 0.004191 |
| *Ntf3* | Neurotrophin 3 | 0.420199255 | 0.038876 |
| *Fam174b* | Family with sequence similarity 174, member B | 0.414689246 | 0.037572 |
| *Slc26a10* | Solute carrier family 26, member 10 | 0.394412144 | 0.022697 |
| *Pla2r1* | Phospholipase A2 receptor 1 | 0.354842083 | 0.048858 |
| *Gpc1* | Glypican 1 | 0.354767031 | 0.022697 |
| *Rnase4* | Ribonuclease, RNase A family 4 | 0.321464634 | 0.043595 |
| *Olfm1* | Olfactomedin 1 | 0.276916317 | 0.036956 |
| *Cd209b* | CD209b antigen | 0.268887021 | 0.009718 |
| *Flrt1* | Fibronectin leucine rich transmembrane protein 1 | 0.200652325 | 0.048858 |
| *Ada* | Adenosine deaminase | 0.153417209 | 0.022697 |

*Genes are displayed that were significantly up- or downregulated when compared to $Ctnnb1^{WT}$ controls with Fold change (FC) < 1 or > 1. Adjusted p-values were calculated for the differences of means of log10 of expression values between $Ctnnb1^{OE-EC}$ and $Ctnnb1^{WT}$.*

in the Hallmark gene sets. Among the most regulated gene sets, we found "Myc targets V1 and V2" and "Cholesterol homeostasis" (**Figure 4A**) followed by "G2M Checkpoint" and "E2F targets." Furthermore, GSEA confirmed the activation of Wnt/β-catenin signaling in β-catenin GOF mutation in LSECs (**Figure 4A**). Overrepresentation analysis (ORA) of the significantly dysregulated genes by using Enrichr revealed significant alterations in the gene ontology (GO) biological processes 2018 library (**Figure 4B**). "Positive regulation of cell differentiation" was identified as the most significant GO term in LSECs with β-catenin GOF mutation (**Figure 4B**).

An established panel of LSEC-associated and CEC-associated marker genes (Geraud et al., 2010, 2017; Olsavszky et al., 2020) was analyzed in LSECs isolated from $Ctnnb1^{WT}$ control and $Ctnnb1^{OE-EC}$ animals. Gene expression analysis pointed out that β-catenin GOF mutation in LSECs of $Ctnnb1^{OE-EC}$ mice dysregulated LSEC- and CEC-associated genes (**Figure 4C**). GSEA revealed a significant induction of a CEC-associated gene set (**Figure 4D**) and a significant loss of an LSEC gene set (**Figure 4E**). As Wnt-/β-catenin signaling is a well-known driver for brain endothelial differentiation (Liebner et al., 2008), we hypothesized that β-catenin signaling activation in LSECs might result in partial brain EC reprograming. When performing GSEA with a brain endothelial gene set, which was generated by comparing published single-cell RNA-seq data from brain vs. liver ECs (Sabbagh et al., 2018), a significant enrichment for brain EC transcripts was found in $Ctnnb1^{OE-EC}$ LSEC (**Figure 4F**). Among the genes that were significantly upregulated in $Ctnnb1^{OE-EC}$ LSEC with a fold-change of >2, several genes could be detected that were also highly expressed in brain ECs (**Table 2**). As the expression of TJ molecule, Cldn5 was previously shown to be upregulated by endothelial Wnt-/β-catenin GOF in the leaky suprafornical organ (Benz et al., 2019), we compared

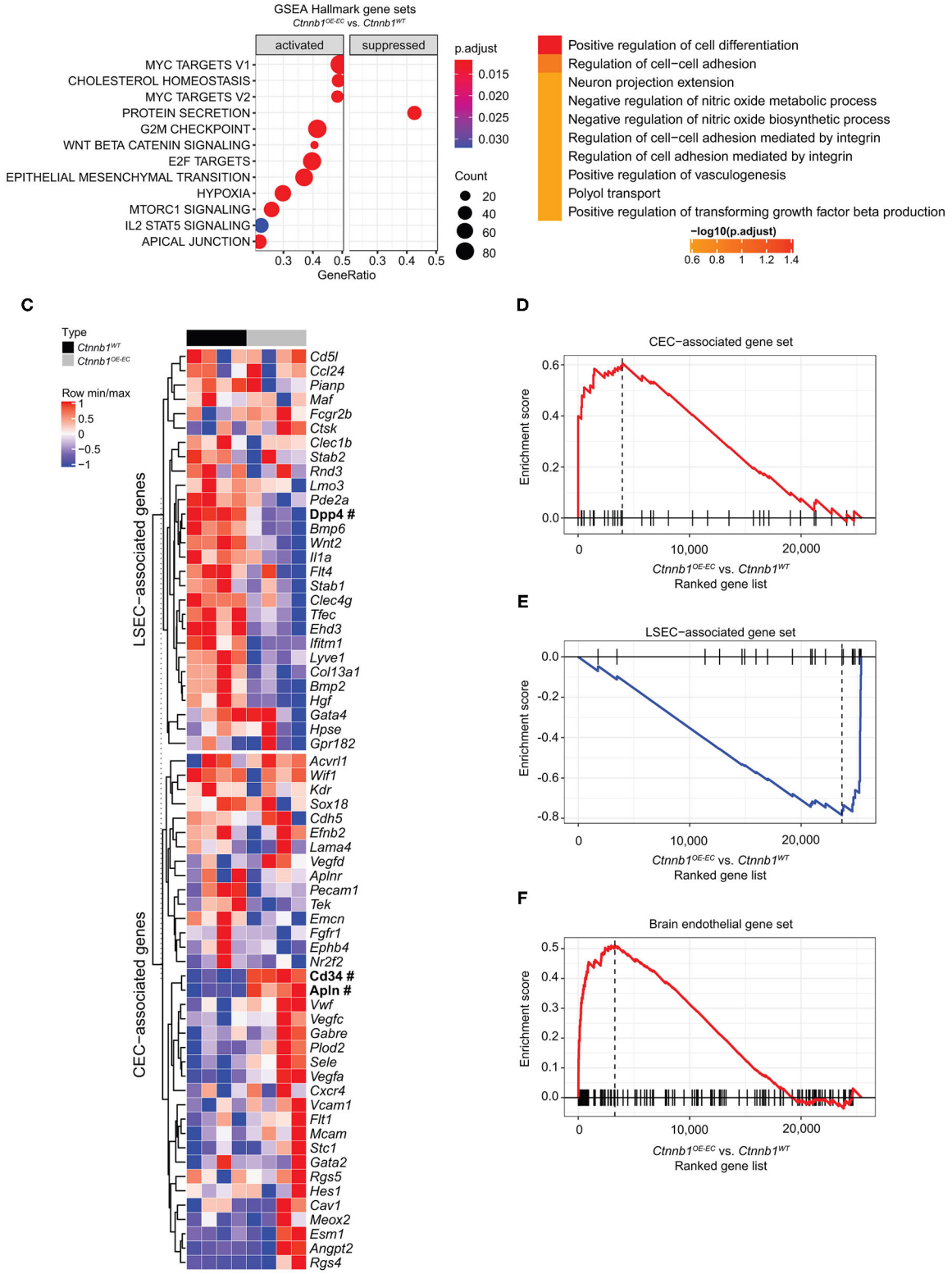


**FIGURE 4 |** Hepatic endothelial *Ctnnb1* overactivation causes sinusoidal transdifferentiation. **(A)** Gene Set Enrichment Analysis-Kyoto Encyclopedia of Genes and Genomes (GSEA-KEGG) pathway alterations analyzed using MSigDB hallmark gene sets in freshly isolated LSECs from 2-month-old female $Ctnnb1^{WT}$ and $Ctnnb1^{OE-EC}$ ($n = 4$). **(B)** Overrepresentation analysis of gene ontology "biological processes" library. **(C)** Heat map of the liver sinusoidal endothelial cell (LSEC)- and

*(Continued)*

**FIGURE 4 |** continuous endothelial cell (CEC)-associated genes. Selected genes are shown for isolated LSECs from *Ctnnb1*$^{WT}$ (black) and *Ctnnb1*$^{OE-EC}$ mice (gray). Significant samples are written in bold and marked with # ($n = 4$ samples per group). The heat map color represents the mean and maximum values for each gene. The intensity scale of the standardized expression values ranges from dark blue (low expression) to dark red (high expression). Enrichment plots of **(D)** LSEC-associated ($p = 0.0023$; NES = −2.43) and **(E)** CEC-associated ($p = 0.0023$; NES = 2.04) genes ($n = 4$). **(F)** Enrichment plots of brain endothelial genes ($p = 0.0001$; NES = 2.16) ($n = 4$).

**TABLE 2 |** Brain endothelial transcripts.

| Gene symbol | Gene title | Fold change *Ctnnb1*$^{OE-EC}$ > *Ctnnb1*$^{WT}$ | Adjusted p-value for Diff of genotype = *Ctnnb1*$^{OE-EC}$–*Ctnnb1*$^{WT}$ |
|---|---|---|---|
| *Slc35f2* | Solute carrier family 35, member F2 | 35.13809 | 0.000397 |
| *Myo1b* | Myosin IB | 6.942870328 | 0.023473 |
| *Tcf7* | Transcription factor 7, T cell specific | 4.800597937 | 0.013887 |
| *Axin2* | Axin 2 | 4.416998695 | 0.022697 |
| *Pla2g16* | Phospholipase A2, group XVI | 4.319974957 | 0.039368 |
| *Il17ra* | Interleukin 17 receptor A | 3.86500417 | 0.031256 |
| *Extl3* | Exostoses (multiple)-like 3 | 3.381491933 | 0.037503 |
| *Ptprg* | Protein tyrosine phosphatase, receptor type, G | 2.158891183 | 0.022697 |
| *Cachd1* | Cache domain containing 1 | 2.065569075 | 0.007219 |

*Genes are displayed that were significantly upregulated in Ctnnb1*$^{OE-EC}$ *LSEC compared to Ctnnb1*$^{WT}$ *LSEC with a fold change (FC) > 2 and also overexpressed in brain endothelial cells compared to liver endothelial cells (Daneman et al., 2010; Sabbagh et al., 2018).*

the expression levels of Cldn5 in *Ctnnb1*$^{OE-EC}$ and control liver. Expression of Cldn5 was not altered in *Ctnnb1*$^{OE-EC}$ compared with control LSECs (**Supplementary Figure 5A**).

In addition, the expression of markers for endothelial zonation, that is, Emcn and LYVE1 (Walter et al., 2014) were analyzed. A significant loss of mid-zonal LSEC marker LYVE1 was found, indicating disturbed endothelial liver zonation (**Figures 5A,B**). However, the expression of pericentral LSEC and CEC marker Emcn was not altered on protein level (**Figure 5A**). Despite disturbed endothelial zonation, the expression of pan-endothelial marker podocalyxin or CD31 was unaltered indicating no major changes in vascular density in *Ctnnb1*$^{OE-EC}$ livers (**Supplementary Figure 5B**). Furthermore, β-catenin GOF in the LSECs did not alter the expression of LSEC marker CD32b (**Supplementary Figure 5C**), LSEC scavenger receptors *Stab1* and *Stab2* (**Supplementary Figures 5D,E**) or CEC markers *Vegfr2*, Caveolin-1, ICAM1, vascular cell adhesion molecule (VCAM), or VE-cadherin (**Supplementary Figures 5C, 6A–C**).

To confirm the transcriptomic alterations seen in *Ctnnb1*$^{OE-EC}$ LSEC, we performed immunofluorescent staining, real-time quantitative PCR (qRT-PCR), and ISH for selected EC genes and proteins. The selection was either based on significant regulation among the list of CEC-associated genes (**Figure 4C**), relation to liver fibrosis [*Gata4*, *Myc*, platelet-derived growth factor subunit B (*Pdgfb*)] (Winkler et al., 2021), or established LSEC angiocrine factors. Upon ISH, the expression of the bone morphogenetic protein (*Bmp*) 2 was not significantly altered (**Figures 5C,D**). This was in line with Prussian blue staining of the liver, which did not show iron deposition in the liver of *Ctnnb1*$^{OE-EC}$ mice (**Supplementary Figure 4B**). Moreover, *Hamp* expression in liver lysates was unaltered, indicating preserved BMP2–HAMP signaling (**Supplementary Figure 4D**). While LSEC angiocrine factor *Wnt2* was significantly downregulated, *Hgf* and *Wnt9b* were not significantly altered (**Figures 5C,D**). β-catenin GOF mutation in LSECs did not alter the expression of LSEC master regulator GATA4 on protein or mRNA level (**Figure 5E**) or pro-fibrotic angiocrine factor *Pdgfb* (**Figure 5F**). On the contrary, transcription factor *Myc* and CEC markers *CD34* and *Apln* were significantly upregulated in *Ctnnb1*$^{OE-EC}$ LSEC (**Figures 4C**, **5F–H**).

## DISCUSSION

Our data show that imbalanced or overactivated β-catenin signaling in LSECs leads to sinusoidal transdifferentiation, including dysregulated lipid homeostasis. Reduced overall survival of *Ctnnb1*$^{OE-EC}$ mice was most likely independent from LSEC transdifferentiation and dysregulated lipid homeostasis, but rather resulted from progressive heart dysfunction. The heart phenotype observed in *Ctnnb1*$^{OE-EC}$ mice is comparable to β-catenin GOF mutation studies in arterial ECs by using a *Bmx-CreER*$^{T2}$ mice, although reporter activity in heart ECs of *Clec4g-iCre;R26YFP* mice was identified in more than just arterial ECs, namely in heart capillaries, endocardium, and venous coronary vessels. Mechanistically, activation of Wnt-/β-catenin signaling in arterial ECs of the heart was shown to result in progressive heart failure through suppressing neuregulin-ErbB signaling (Nakagawa et al., 2016).

In the liver, β-catenin GOF mutation in LSECs resulted in sinusoidal-to-continuous transdifferentiation with downregulation of midzonal LSEC marker LYVE1 and angiocrine factor *Wnt2*, and upregulation of CEC markers *CD34* and *Apln*. This rather "mild" capillarization program lacking HSC activation and perisinusoidal extracellular matrix

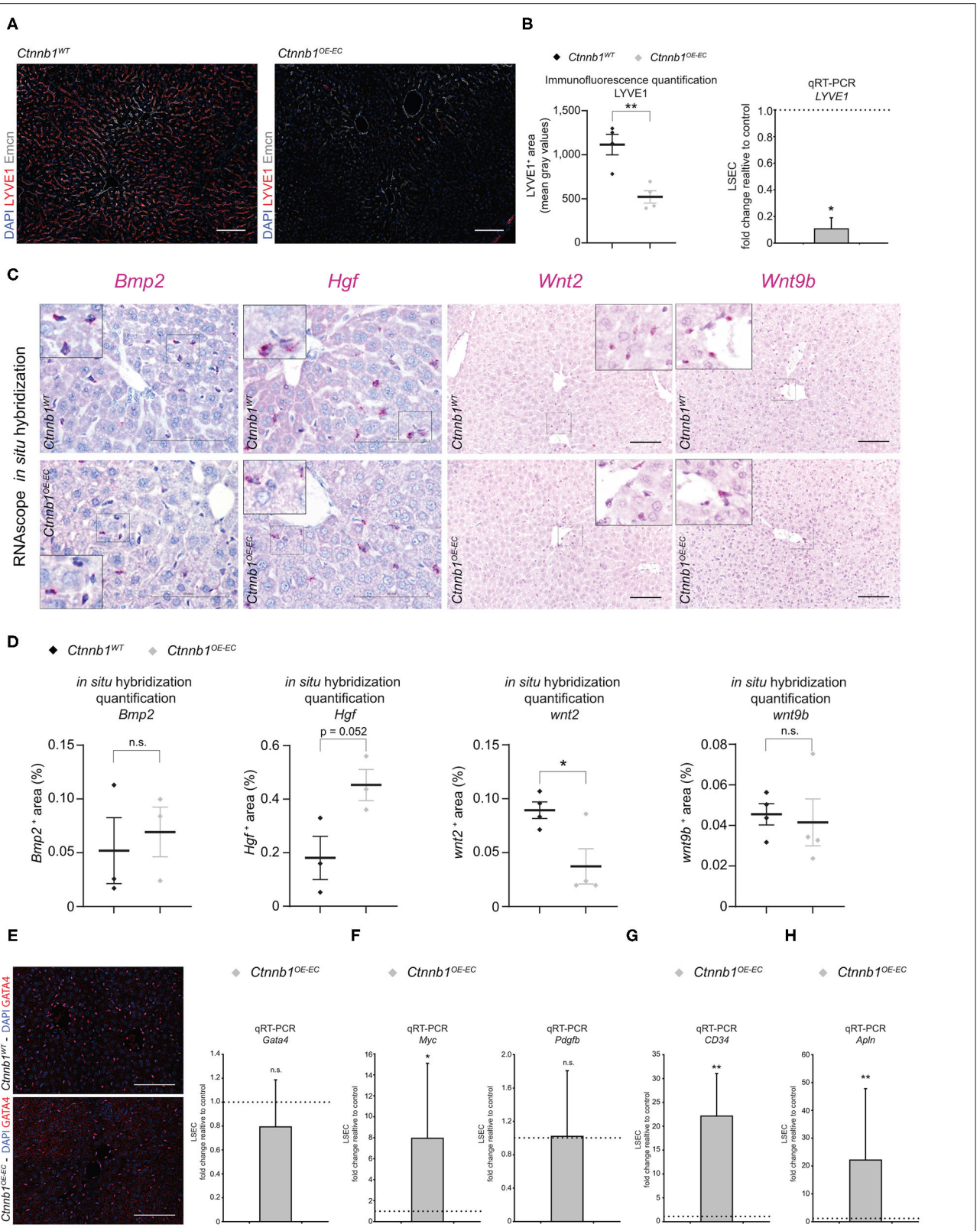


**FIGURE 5 |** Hepatic endothelial *Ctnnb1* overactivation causes loss of LSEC-associated genes and induction of CEC and brain EC genes. **(A)** IF staining of DAPI, LYVE1, and Emcn in the liver of 2- to 3-month-old female *Ctnnb1*$^{WT}$ and *Ctnnb1*$^{OE-EC}$ mice ($n = 4$). Scale bar 100 μm. **(B)** Left panel: IF quantification of LYVE1$^+$ area. Results are represented as mean ± SEM. $^{**}p < 0.01$. Right panel: qRT-PCR for *LYVE1* of cDNA from *Ctnnb1*$^{OE-EC}$-LSECs compared to *Ctnnb1*$^{WT}$ control

*(Continued)*

**FIGURE 5 |** LSECs ($n = 4$). β-Actin was used as housekeeping gene. *$p < 0.05$. **(C)** *Bmp2*, *Hgf*, *Wnt2*, *Wnt9b* mRNA RNAScope *in situ* hybridization assay of 2- to 3-month-old female $Ctnnb1^{WT}$ and $Ctnnb1^{OE-EC}$ mice liver sections ($n \geq 3$). Scale bar 100 μm. **(D)** Quantification of *Bmp2*, *Hgf*, *Wnt2*, *Wnt9b* mRNA RNAScope *in situ* hybridization assay. Results are represented as mean ± SEM. ns, not significant. *$p < 0.05$. **(E)** Left panel: IF staining of DAPI and GATA4 in the liver of 2- to 3-month-old female $Ctnnb1^{WT}$ and $Ctnnb1^{OE-EC}$ mice ($n = 4$). Scale bar 100 μm. Right panel: qRT-PCR for *Gata4* with cDNA from $Ctnnb1^{OE-EC}$-LSECs compared to $Ctnnb1^{WT}$ control LSECs ($n = 4$). β-Actin was used as housekeeping gene. n.s., not significant. **(F)** qRT-PCR for *Myc* and *Pdgfb* with cDNA from $Ctnnb1^{OE-EC}$-LSECs compared with $Ctnnb1^{WT}$ control LSECs ($n = 3$). β-Actin was used as housekeeping gene. *, $p < 0.05$; n.s., not significant. **(G)** qRT-PCR for *CD34* with cDNA from $Ctnnb1^{OE-EC}$-LSECs compared with $Ctnnb1^{WT}$ control LSECs ($n = 3$). β-Actin was used as housekeeping gene. **$p < 0.01$. **(H)** qRT-PCR for *Apln* with cDNA from $Ctnnb1^{OE-EC}$-LSECs compared with $Ctnnb1^{WT}$ control LSECs ($n = 3$). β-Actin was used as housekeeping gene. **$p < 0.01$.

deposition did not result in hepatopathy or liver fibrosis. Interestingly, Wnt-target gene *Myc* (He et al., 1998) was significantly induced in $Ctnnb1^{OE-EC}$ LSECs. Previous work by us could show, that loss of LSEC master regulator GATA4 also induced pro-angiogenic *Myc* in LSECs, to further amplify a pro-fibrotic angiocrine program, including *de novo Pdgfb* expression, resulting in perisinusoidal liver fibrosis (Winkler et al., 2021). β-catenin GOF in LSECs did not significantly regulate GATA4 expression, which most likely protects against a complete capillarization program and perisinusoidal liver fibrosis by suppressing pro-fibrotic angiocrine factors such as *Pdgfb*, which was unaltered in $Ctnnb1^{OE-EC}$ LSEC despite a significant *Myc* induction.

Angiocrine Wnt-signaling is vital for liver growth and metabolic liver zonation and Wnt-signaling in LSECs is linked to autocrine growth effects (Klein et al., 2008; Geraud et al., 2010; Leibing et al., 2018). While activation of β-catenin in LSECs reduced angiocrine *Wnt2*, this reduction together with unaltered *Wnt9b* was not sufficient to impair metabolic liver zonation in $Ctnnb1^{OE-EC}$ mice. Interestingly, EC proliferation was significantly induced by activation of β-catenin in LSECs. These findings are supported by GSEA results of $Ctnnb1^{OE-EC}$ LSEC with enrichment in the gene sets "G2M Checkpoint" and "E2F Targets," both resembling a pro-proliferative state, thereby indicating that β-catenin overactivation in LSECs stimulates endothelial proliferation. This is in line with data observed in postnatal brain and retina, showing that deficiency of endothelial β-catenin signaling impairs endothelial proliferation and sprouting (Martowicz et al., 2019).

Notably, activation of β-catenin in LSECs resulted in the upregulation of genes that are known to be expressed by the brain ECs (Daneman et al., 2010; Wang et al., 2019) and GSEA could confirm the enrichment of brain EC transcripts (Sabbagh et al., 2018) in $Ctnnb1^{OE-EC}$ LSECs. In contrast to LSEC, that belong to discontinuous sinusoidal ECs which enable transfer of fluids, nutrients, and small solutes through open fenestrations within the sinusoidal wall (Wisse et al., 1985; Augustin and Koh, 2017), the brain ECs belong to the group of CECs, expressing specialized TJ molecules and transporters for restricting paracellular passage and transcellular trafficking, thereby generating the tightly sealed blood–brain barrier (Langen et al., 2019). In line with our results, ectopic β-catenin signaling activation in the highly permeable and fenestrated vasculature of the circumventricular organs is sufficient for BBB reprograming (Benz et al., 2019; Wang et al., 2019). Furthermore, inducible pan-endothelial Ctnnb1 GOF showed some overlap with genes dysregulated in $Ctnnb1^{OE-EC}$ LSEC despite using different Cre lines (Munji et al., 2019). Vice-versa, loss of Wnt-signaling activity impairs brain endothelial differentiation by downregulating TJ molecules and transporter proteins, while increasing the expression of the plasmalemma vesicle–associated protein (PLVAP) (Liebner et al., 2008; Stenman et al., 2008; Daneman et al., 2009). However, the expression of TJ molecule Cldn5 was not enhanced in $Ctnnb1^{OE-EC}$ LSEC, which could be a result of maintained expression of LSEC master regulator GATA4. Notably, PLVAP knockout mice developed a reduction of LSEC fenestrations, which led to elevated serum levels of triglycerides, low-density lipoprotein, and cholesterol due to retention of chylomicron remnants in the blood. The authors speculated that compensatory hepatocyte *de novo* lipogenesis was responsible for steatosis, steatohepatitis, and liver fibrosis (Herrnberger et al., 2014). $Ctnnb1^{OE-EC}$ mice neither showed liver steatosis nor fibrosis, which argue against reduced PLVAP expression as a main driver of isolated hypertriglyceridemia in $Ctnnb1^{OE-EC}$ mice.

As only microvascular ECs in the liver with sinusoidal differentiation allow filtration of chylomicron remnants from the blood (Fraser et al., 1995; Cogger et al., 2006), β-catenin-mediated transdifferentiation of liver sinusoids with partial BBB reprograming in $Ctnnb1^{OE-EC}$ mice may impair uptake of chylomicrons and subsequently lead to elevated serum lipid levels. However, these metabolic alterations are in contrast with previous results, showing that neither sinusoidal capillarization with loss of fenestrations and formation of a basement membrane in Gata4-deficient LSEC ($Gata4^{LSEC-KO}$), nor partial sinusoidal capillarization/trandifferentiation in mice with enhanced Notch signaling in LSECs ($NICD^{OE-HEC}$) are associated with reduced levels of serum cholesterol and triglycerides (Wohlfeil et al., 2019; Winkler et al., 2021). This argues against a general impairment of lipid transfer into the space of Disse during sinusoidal capillarization/transdifferentiation and indicates that hypertriglyceridemia in $Ctnnb1^{OE-EC}$ mice is a result of β-catenin-mediated LSEC transdifferentiation by impaired transendothelial transport mechanisms and/or by altered angiocrine signaling that control hepatocyte lipogenesis/lipolysis.

Among the *de novo* expressed genes in $Ctnnb1^{OE-EC}$ LSEC, *Apln* was found as the second most upregulated gene. Apelin (APLN) is a secreted peptide, which is widely expressed in different cell types, including CECs and is also known as a regulator of transendothelial lipid transport (Hwangbo et al., 2017). Mice with *Apln* knockout become obese and show more fat deposition as a consequence of increased vascular permeability with greater uptake of fatty acids. On the other hand, transgenic *Apln* mice that express apelin under the

transcriptional control of the keratin 14 promoter are protected from obesity and show a reduced endothelial permeability (Sawane et al., 2011, 2013). Interestingly, Huang and colleagues were able to show that also Apln signaling in hepatocytes protects against lipid accumulation in the liver (Huang et al., 2017). As the promoter region of the *Apln* gene has transcription factor–binding sites for Wnt signaling downstream targets *Tcf/Lef* (Chen et al., 2019), *Apln* expression might be transcriptionally activated by Wnt-β-catenin signaling activation in $Ctnnb1^{OE-EC}$ LSECs. This is in line with silencing experiments of β-catenin in pulmonary ECs showing that *Apln* mRNA and protein expression were reduced (Alastalo et al., 2011). Thus, in $Ctnnb1^{OE-EC}$ mice *de novo Apln* expression in transdifferentiated LSECs may be involved in dysregulated lipid homeostasis. Yet, one has to consider that aberrant *Apln* expression is also found in $CD34^{+}$ capillarized LSECs in liver fibrosis (Winkler et al., 2021) and cirrhosis (Yokomori et al., 2012) and also pro-angiogenic effects were similar to the vascular apelin signaling (Helker et al., 2020).

Together, normal sinusoidal differentiation is decisive for the fulfillment of the typical LSEC functions such as scavenging, immunoregulation, protection against stellate cell activation, and fibrosis, but also for the angiocrine regulation of liver regeneration and iron metabolism (Poisson et al., 2017; Shetty et al., 2018; Lafoz et al., 2020; Koch et al., 2021). While endothelial Wnt-signaling activity is largely confined to brain ECs for the maintenance of the BBB (Sabbagh et al., 2018), here we can show for the first time that low-level liver endothelial Wnt-signaling *in vivo* is crucial for maintaining sinusoidal differentiation, which is required for regulation of proper hepatic lipid metabolism. Further research is necessary to analyze the specific contributions of LSECs in hepatic fat absorption and metabolism. Future work will have to address which angiocrine signaling pathways may be involved in this process, extending the knowledge that liver endothelial fatty acid absorption is not mainly a passive mechanism mediated by open fenestrations in LSECs. This is of particular interest as dyslipidemia is a major risk factor for cardiovascular disease.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE175777.

## ETHICS STATEMENT

The animal study was reviewed and approved by Regional Council Karlsruhe. Written informed consent was obtained from the owners for the participation of their animals in this study.

## AUTHOR CONTRIBUTIONS

P-SK, KSa, SG, and VO: study concept and design. P-SK, KSa, JHeil, CDS, SK, JHo, MW, CT, CS, KSc, MT, FT, JHein, CG, SG, and VO: experimental work, analysis, and interpretation of data. P-SK, KSa, and VO: writing original draft. All authors writing and reviewing the manuscript before submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2021.722394/full#supplementary-material

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REVIEW
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# The Scavenger Function of Liver Sinusoidal Endothelial Cells in Health and Disease

*Sabin Bhandari†, Anett Kristin Larsen†, Peter McCourt, Bård Smedsrød* and Karen Kristine Sørensen*

*Vascular Biology Research Group, Department of Medical Biology, University of Tromsø (UiT) – The Arctic University of Norway, Tromsø, Norway*

The aim of this review is to give an outline of the blood clearance function of the liver sinusoidal endothelial cells (LSECs) in health and disease. Lining the hundreds of millions of hepatic sinusoids in the human liver the LSECs are perfectly located to survey the constituents of the blood. These cells are equipped with high-affinity receptors and an intracellular vesicle transport apparatus, enabling a remarkably efficient machinery for removal of large molecules and nanoparticles from the blood, thus contributing importantly to maintain blood and tissue homeostasis. We describe here central aspects of LSEC signature receptors that enable the cells to recognize and internalize blood-borne waste macromolecules at great speed and high capacity. Notably, this blood clearance system is a silent process, in the sense that it usually neither requires or elicits cell activation or immune responses. Most of our knowledge about LSECs arises from studies in animals, of which mouse and rat make up the great majority, and some species differences relevant for extrapolating from animal models to human are discussed. In the last part of the review, we discuss comparative aspects of the LSEC scavenger functions and specialized scavenger endothelial cells (SECs) in other vascular beds and in different vertebrate classes. In conclusion, the activity of LSECs and other SECs prevent exposure of a great number of waste products to the immune system, and molecules with noxious biological activities are effectively "silenced" by the rapid clearance in LSECs. An undesired consequence of this avid scavenging system is unwanted uptake of nanomedicines and biologics in the cells. As the development of this new generation of therapeutics evolves, there will be a sharp increase in the need to understand the clearance function of LSECs in health and disease. There is still a significant knowledge gap in how the LSEC clearance function is affected in liver disease.

**Keywords: blood clearance, liver, sinusoid, endothelial cell (EC), scavenger receptor, mannose receptor, Fc-gamma receptor IIb, scavenger endothelial cells**

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****Correspondence:***
*Bård Smedsrød*
*bard.smedsrod@uit.no*

*† These authors have contributed equally to this work and share first authorship*

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**Abbreviations:** acLDL, acetylated low density lipoproteins; AGE, advanced glycation end-products; FcγRIIb2, Fc-gamma receptor IIb2; FSA, formaldehyde-treated serum albumin; HCC, hepatocellular carcinoma; LSEC, liver sinusoidal endothelial cell; LSECtin, liver and lymph node sinusoidal endothelial cell C-type lectin; L-SIGN, liver/lymph node-specific ICAM-3 grabbing non-integrin; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1; NPC, non-parenchymal liver cells; oxLDL, oxidized low density lipoprotein; RES, reticuloendothelial system; SEC, scavenger endothelial cell; scRNA-seq, single cell RNA sequencing; SR, scavenger receptor; tPA, tissue plasminogen activator; VLP, virus-like particle.

## INTRODUCTION

The aim of the present review is to give an outline of the blood clearance function of the mammalian liver sinusoidal endothelial cells (LSECs), which constitute one of the two cellular arms of the hepatic reticuloendothelial system (RES). It is generally accepted today that the hepatic RES consists of two types of specialized clearance cells, namely the liver macrophages, or Kupffer cells, that are geared to take up particles (>200 nm) *via* phagocytosis, and the non-phagocytic LSECs that are specially equipped for clearance of macromolecules and colloids by receptor-mediated endocytosis (Seternes et al., 2002). This understanding is the result of a scientific evolution that has taken place over more than a century, starting with the discovery of the macrophage (Metchnikoff, 1884, 1968), and the use of vital stains to locate the anatomical sites of uptake of blood-borne exogenous and endogenous waste material (Kiyono, 1914; Aschoff, 1924). Uptake of vital stains (a type of colloidal particles) occurred in so-called "reticuloendothelial cells" (Aschoff, 1924), which are endothelial cells with high scavenging activity (Seternes et al., 2002). Readers who wish to look deeper into the historical backdrops and the scientific evolution of the development of the RES concept are referred to Smedsrød (2004) and Sørensen et al. (2012).

A series of experiments during the 1980s established that soluble macromolecules and nanoparticles of various kinds were rapidly cleared from the circulation of mammals mainly by specialized endothelial cells in the liver sinusoids, with negligible uptake in the Kupffer cells (Smedsrød et al., 1990b). Violating the paradigm at the time, that the Kupffer cells alone constituted the RES (Van Furth et al., 1972), these findings came as a surprise. We know today that the LSECs are characterized by a remarkably active receptor-mediated endocytosis making them an important part of the hepatic RES (Smedsrød et al., 1990b; Sørensen et al., 2012).

## Tissue Turnover Processes and Waste Clearance

The story about LSECs and other scavenger endothelial cells (SECs) is largely about how the body deals with own and foreign waste products. The metabolic processes in our tissues and cells generate a constant release of all kinds of biological macromolecules. For instance, our connective tissues continuously release considerable amounts of large fragments of matrix macromolecules, such as collagens, procollagen propeptides, and connective tissue polysaccharides, e.g., hyaluronan and chondroitin sulfate proteoglycans. A small portion of these molecules are endocytosed and degraded by local connective tissue cells, whereas the majority are transported with lymph to the lymph nodes, where specialized cells scavenge them (Laurent et al., 1986a; Østgaard et al., 1995; Fraser et al., 1997). The proportion that escapes clearance in lymph nodes are released to the general circulation, where they are finally effectively cleared and degraded by the LSECs (Smedsrød et al., 1985a, 1989, 1990a; Laurent et al., 1986a; Smedsrød, 1988, 1990; Melkko et al., 1994; Østgaard et al., 1995; Malovic et al., 2007; **Figure 1** and **Table 1**). Of note, bone lacks lymph capillaries, and the large amounts of collagen and waste from collagen production that are released from bone tissue are released directly to the blood circulation. Thanks to the LSEC scavenger and mannose receptors these molecules are very effectively removed from the circulation. A different group of waste products that must be removed rapidly from the circulation include the powerful fibrinolytic tissue plasminogen activator (tPA), which is cleared mainly by the LSEC mannose receptor, and to a lesser extent by the galactose receptor of hepatocytes (Smedsrød and Einarsson, 1990). LSECs also participate in elimination of circulating small soluble immune complexes *via* the Fc-gamma receptor IIb2 (FcγRIIb2) (Mousavi et al., 2007). Moreover, macromolecules released from cells under normal or pathophysiological conditions (e.g., lysosomal enzymes and poly- and oligonucleotides) are effectively cleared from the circulation by LSECs (Martin-Armas et al., 2006; Elvevold et al., 2008a) (reviewed in Sørensen et al., 2015). The receptors involved and the speed of clearance observed with several of the waste macromolecules that are eliminated by LSECs are presented in **Table 1** and will also be dealt with in more detail in the following sections.

## Clearance of Virus and Other Nanoparticles From the Circulation

In addition to their significant function of removing endogenous waste material, LSECs also play a role in blood clearance of exogenous ligands such as virus and other nanoparticles. Studies challenging mice with intravenous administration of adenovirus (Ganesan et al., 2011), BK and JC polyomavirus-like particles (VLPs) (Simon-Santamaria et al., 2014) and human immunodeficiency virus (HIV)-VLPs (Mates et al., 2017) showed a rapid and efficient clearance from blood with liver being the main responsible organ and with high uptake in LSECs. Liver was also found to be the main organ for clearing simian immunodeficiency virus in Rhesus monkeys (Zhang et al., 1999). The hepatic clearance was predominantly in LSECs with approximately 90% of eliminated blood-borne adenovirus or HIV-VLPs associated with this cell type, while the remaining associated with Kupffer cells (Ganesan et al., 2011; Mates et al., 2017). Mates and coworkers calculated that the liver sinusoids possessed an astonishing clearance rate of more than 100 million HIV-VLPs per minute (Mates et al., 2017). *In vitro* experiments have also shown that rat LSECs endocytose and degrade T4 bacteriophages (Øie et al., 2020). This efficient viral uptake suggests that LSECs may have an important role in the innate immune defense against viral infections. The receptors responsible for viral endocytosis in LSECs are not yet identified. Other receptors expressed by LSECs (L-SIGN, liver/lymph node-specific ICAM-3 grabbing non-integrin; and LSECtin, liver and lymph node sinusoidal endothelial cell C-type lectin) have been shown to interact with surface glycoproteins of Ebola virus, HIV, SARS coronavirus (CoV), and hepatitis C virus (HCV) (Shetty et al., 2018), and recently with SARS-CoV-2

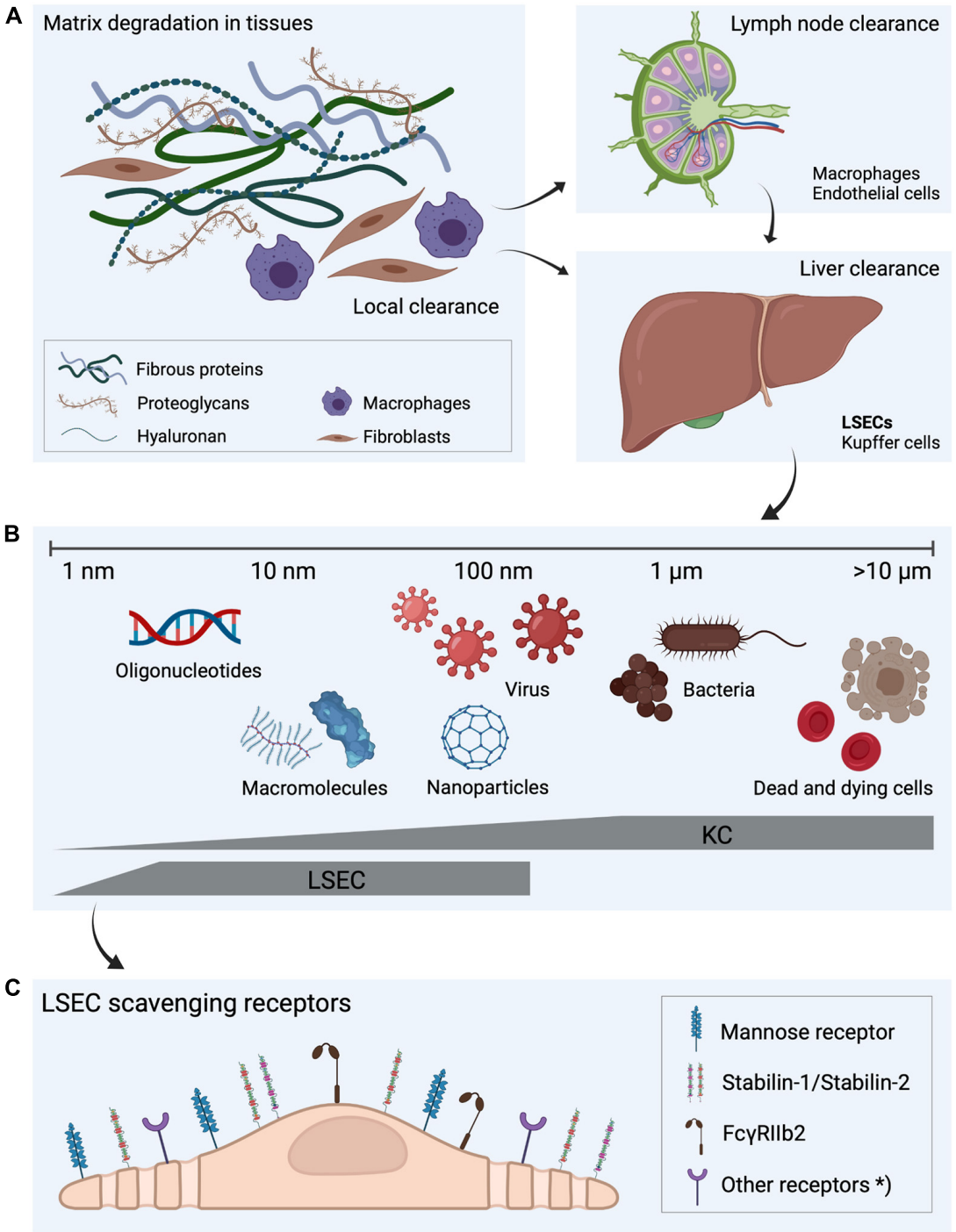


**FIGURE 1 |** Fate of extracellular matrix turnover products, the dual cell principle of waste clearance and the role of liver scavenger cells in waste clearance. **(A)** Molecular fragments are continuously released during the constant turnover of the extracellular matrix. Some of the degradation products are digested locally but a large proportion is drained to lymph nodes where they are endocytosed by macrophages and sinusoidal endothelial cells (Laurent et al., 1986a; Fraser et al., 1997). The fragments that escape uptake in lymph node cells leak to the blood circulation (Østgaard et al., 1995), and are removed from blood by endocytosis in liver scavenger cells. **(B)** Liver sinusoidal endothelial cells (LSECs) and Kupffer cells, which together make up the largest population of scavenger cells in the body, share the scavenging workload in the liver (Seternes et al., 2002). LSECs are specialized on effective clathrin-mediated endocytosis of soluble macromolecules and nanoparticles, whereas larger particles, such as bacteria and dead and dying cells are cleared by the Kupffer cells, illustrating "the dual cell principle of waste clearance" (Sørensen et al., 2012). **(C)** The uptake of soluble macromolecules in LSECs are mediated by a range of endocytic receptors, with the mannose receptor, stabilin-1, stabilin-2, and FcγRIIb2 being the most investigated. *Other endocytic receptors may also contribute to the effective waste clearance performed by LSECs. Figure created with BioRender.com.

**TABLE 1 |** Tissue turnover products cleared from blood mainly by LSECs*, the endocytosis receptor involved in the LSEC uptake, examples of rate of blood clearance of ligands taken up by LSECs following i.v. administration of the ligand, and species examined.

| Ligand | LSEC receptor | References | Examples of injected material (dose, inj. site) | Species | Decay of plasma/blood radioactivity (% eliminated) | References |
|---|---|---|---|---|---|---|
| Hyaluronan | Stabilin-2[a] | Smedsrød et al., 1984; McCourt et al., 1999; Zhou et al., 2000; Politz et al., 2002 | [$^3$H]-hyaluronan (30–32 μg, marginal ear vein) | Rabbit | $t_{1/2}$ = 2.5–4.5 min (88% uptake in liver at 19 min after injection) | Fraser et al., 1981 |
| | | | $^{125}$I-tyramine cellobiose (TC)-labeled hyaluronan (MW = 2.5 × $10^5$) (tail vein) | Rat | $t_{1/2}\alpha$ = 0.9 min (79% uptake in liver at 30 min after injection) | Dahl et al., 1988 |
| | | | [$^3$H]-hyaluronan (60–130 μg, cubital vein) | Human | $t_{1/2}$ = 2.6-5.5min (90% was eliminated from blood after 10 min) | Fraser et al., 1984 |
| Chondroitin sulfate | Stabilin-2[a] | Smedsrød et al., 1985b; Harris and Weigel, 2008 | [$^3$H]-chondroitin sulfate (CS) and $^{125}$I-CS proteoglycan | Rat | Clearance rate not examined but the main uptake was in LSECs | Smedsrød et al., 1985b |
| Heparin | Stabilin-2[b] | Harris et al., 2008, 2009; Øie et al., 2008 | $^{125}$I-FITC-labeled unfractionated heparin (0.1 IU/kg, tail vein) | Rat | $t_{1/2}$ = 1.71 min (71% was recovered in liver after 15 min) | Øie et al., 2008 |
| Nidogen | SR | Smedsrød et al., 1989 | $^{125}$I-TC-nidogen (trace amounts, tail vein) | Rat | $t_{1/2}$ = 2-3 min (78% was recovered in liver after 1 h) | Smedsrød et al., 1989 |
| Alpha chains of types I–V and XI collagen | Mannose[c] receptor | Smedsrød et al., 1985a; Smedsrød, 1990; Malovic et al., 2007 | $^{125}$I-FITC-labeled heat-denatured collagen (50 μg, tail vein) | Rat | $t_{1/2}\alpha$ = 0.8 min (75%) $t_{1/2}\beta$ = 3.7 min (25%) | Hellevik et al., 1996 |
| | | | $^{125}$I-DTAF-collagen (heat-denatured) (0.04 mg/kg, tail vein) | Mouse | $t_{1/2}\alpha$ = 0.51 min (90.25%) $t_{1/2}\beta$ = 36.9 min (9.75%) | Malovic et al., 2007 |
| N-terminal propeptide of types I and III procollagen (PINP and PIIINP) | SR. Stabilin-2 | Smedsrød, 1988; Melkko et al., 1994 | $^{125}$I-TC-PINP (5 μg, tail vein) | Rat | $t_{1/2}\alpha$ = 0.59 min (78.5%) $t_{1/2}\beta$ = 3.3 min (21.5%) | Melkko et al., 1994 |
| C-terminal propeptide of type I procollagen (PICP) | Mannose receptor | Smedsrød et al., 1990a | $^{125}$I-TC-PICP (10 μg, tail vein) | Rat | $t_{1/2}$ = 8.7 min | Smedsrød et al., 1990a |
| Tissue plasminogen activator (tPA) | Mannose receptor | Smedsrød and Einarsson, 1990 | $^{125}$I-tPA (1 μg, tail vein) | Rat | $t_{1/2}\alpha$ = 0.6 min (65%) $t_{1/2}\beta$ = 6.4 min (35%) | Smedsrød and Einarsson, 1990 |
| Lysosomal enzymes | Mannose receptor | Hubbard et al., 1979; Isaksson et al., 1983; Elvevold et al., 2008a | $^{125}$I-cathepsin (10 μg, tail vein) | Mouse | $t_{1/2}\alpha$ = 0.9 min (63%) $t_{1/2}\beta$ = 8.9 min (37%) | Elvevold et al., 2008a |
| | | | $^{125}$I-glycosyl asparaginase (trace amounts, tail vein) | Rat | $t_{1/2}\alpha$ = 0.7 min (63%) $t_{1/2}\beta$ = 3.3 min (37%) | Smedsrød and Tollersrud, 1995 |
| | | | $^{125}$I-α-mannosidase (trace amounts, jugular vein) | Pig | $t_{1/2}$ = 5 min (about 60% was recovered in liver, and 18% in lung after 1 h) | Nedredal et al., 2003 |

*(Continued)*

**TABLE 1** | (Continued)

| Ligand | LSEC receptor | References | Examples of injected material (dose, inj. site) | Species | Decay of plasma/blood radioactivity (% eliminated) | References |
|---|---|---|---|---|---|---|
| Formaldehyde-treated serum albumin (FSA)[d] | SR. Stabilin-1, and stabilin2 | Blomhoff et al., 1984; McCourt et al., 1999; Li et al., 2011 | $^{125}$I-FSA (0.1 mg, femoral vein) | Rat | 70% was recovered in LSECs 12 min post injection | Blomhoff et al., 1984 |
| | | | $^{125}$I-FSA (2 µg, tail vein) | Mouse | $t_{1/2}$ = approximately 1–2 min[e] (77% was recovered in liver after 10 min) | Elvevold et al., 2008a |
| | | | $^{125}$I-TC-FSA (trace amounts, jugular vein) | Pig | $t_{1/2}$ = 2 min (about 53% was recovered in liver, and 26% in lung after 1 h) | Nedredal et al., 2003 |

**A more complete overview of macromolecular ligands removed from the circulation by LSECs is presented in Sørensen et al. (2012, 2015). SR, scavenger receptor.*

*[a]The receptor was named the hyaluronan receptor until 1999 when it was found that scavenger receptor (SR) ligands and hyaluronan (HA) bound to the same receptor on LSECs (McCourt et al., 1999). The receptor was later named stabilin-2 (Politz et al., 2002) [aka HARE (Zhou et al., 2000), FEEL-2 (Tamura et al., 2003)].*

*[b]Heparin was found to be a ligand for human stabilin-2 in Harris et al. (2008), and an antibody to the receptor partly inhibited binding of heparin in rat LSECs (Harris et al., 2009) whereas another study in rat did not find heparin binding to stabilin-2 (Øie et al., 2008).*

*[c]Uptake of collagen alpha-chains was until 2007 thought to occur via a distinct collagen receptor in LSECs. In 2007 it was found that this receptor was identical to the mannose receptor (Malovic et al., 2007). Binding of denatured collagen/collagen alpha-chains occur via the fibronectin type II domain in this receptor (Martinez-Pomares et al., 2006; Napper et al., 2006).*

*[d]FSA is a non-physiological ligand that is much used in studies of LSEC function. It is an SR ligand and is internalized both via stabilin-1 and stabilin-2 in LSECs (McCourt et al., 1999; Li et al., 2011).*

*[e]The rate of elimination of $^{125}$I-FSA from blood was not calculated in Elvevold et al. (2008a) but from the decay curve in Figure 5 of that reference we have estimated that that about 50% of the radioactivity in blood at 1 min post injection was eliminated after 2 min and more than 80% was eliminated after 5 min.*

(Kondo et al., 2021). The function of these receptors in LSECs is however, not well known.

## Liver Sinusoidal Endothelial Cell Clearance as a Challenge to Delivery of Nanopharmaceuticals

As outlined in Sørensen et al. (2012) and **Figure 1** LSECs are geared to take up and metabolize several types of macromolecules and nano sized material <200 nm, a size range that includes most types of nanotherapeutics. Although critical for homeostasis maintenance, the powerful capability of LSECs to remove own and foreign substances from the circulation poses a serious challenge for the development of large size/nano pharmaceuticals. Thus, targeting LSECs with nano sized material is clearly a physiological default system, and focus is therefore commonly on finding ways to avoid uptake of nanopharmaceuticals in these cells. The last decades have seen a surge in the development of the new generation nano drugs. Although promising, with the potential to remedy diseases (e.g., cancer, viral infections, and genetic disorders) for which no cure presently exists, the successful development of these compounds are hampered by the lack of understanding of how to achieve control over the hepatic uptake. It is not possible to cover all aspects of the field in this short paragraph. The use of nanoparticles as carriers of RNA therapeutics, and the challenge of controlling liver uptake can serve as an example. For more literature on nanoparticles that are taken up in LSECs, the reader is referred to Kamps et al. (1997), Sigfridsson et al. (2017), Campbell et al. (2018), Hunt et al. (2018).

One reason for using nanocarriers is to protect RNA therapeutics from being degraded by blood plasma RNases following their intravenous administration. Although chemical modifications of oligonucleotides have been developed to make them resistant to degradation in plasma, the problem of uncontrolled LSEC uptake still exists (Godfrey et al., 2017; Shen and Corey, 2018). Renal filtration also contributes importantly by efficient filtration of material smaller than 6 nm (Choi et al., 2007). In addition, uncontrolled accumulation of these compounds may result in hepatotoxic reactions (Godfrey et al., 2017). Hence, siRNA for silencing of gene expression, or mRNA for gene expression are loaded in nanoparticles to carry these oligonucleotides past the LSECs and the liver and bring them intact to the cellular site of their intended therapeutic activity. Much effort is therefore spent to generate nanoparticles that carry therapeutic RNA to the intended cellular site. Out of a plethora of different types of nanoparticles that have been previously tested as vehicles for therapeutic RNA and other drug candidates, it appears that specially designed lipid nanoparticles have particularly attractive properties. This includes ease of manufacture, reduced immune responses, multidosing capabilities, larger payloads, and flexibility of design (Kulkarni et al., 2018). Although much effort is directed toward designing nanoparticles that reach the intended target cells with high precision and enable the RNA cargo to enter the intracellular compartment, the true "elephant in the room," that is uncontrolled clearance by LSECs, is still a serious challenge that must be overcome. A few of those nanoparticle-carried RNA therapeutics that have made it successfully to

the market include gene correction drugs that target the hepatocytes (Roberts et al., 2020). The LSECs allow passage of these nanoparticles (50 nm) through their fenestrae (i.e., open pores of diameter 100–150 nm). Circulating ApoB binds to these lipid nanoparticles, which mediate binding to the hepatocyte low density lipoprotein (LDL) receptor (Akinc et al., 2010). The same authors showed that conjugation of the particle surface with N-acetylgalactosamine (GalNAc), a ligand for the GalNAc receptor [aka asialoglycoprotein receptor, or Ashwell-Morell receptor, (Morell et al., 1971)] that are present on hepatocytes, but not on LSECs, strengthened the uptake of these lipid nanoparticles to the hepatocytes. Despite the success in using lipid nanoparticles as vehicles for transfer of RNA therapeutics to hepatocytes, the difficulty in achieving efficient delivery to target organs and tissues other than the liver is still a major obstacle preventing widespread usage of oligonucleotide therapeutics. One of the keys to solve this problem would be more precise knowledge on how to avoid unwanted uptake in LSECs.

## Factors Contributing to the Effective Blood Clearance Activity in Liver Sinusoidal Endothelial Cells

Nowadays it is widely appreciated that blood clearance is a central physiological function of LSECs. Moreover, there is general agreement that special endocytosis receptors endow LSECs with their scavenger function. Of note, several additional factors must be taken into consideration to explain the role of LSECs as major blood clearance cells (**Table 2**).

The anatomical location clearly plays a role: lining the hundreds of millions of liver sinusoids and covering a total area of approximately 210 $m^2$, i.e., nearly that of a tennis court [Sørensen and Smedsrød (2020); calculated from Blouin et al. (1977)], the LSECs of the human liver are optimally located to effectively survey the large amount of blood that passes every minute. LSECs further make up the largest part of the liver sinusoidal cells, outnumbering the Kupffer cells by about a factor of 2.5 (Pertoft and Smedsrød, 1987).

In addition, a physiological factor contributing to effective interaction of LSECs with the blood is the reduced blood flow through the sinusoids, giving the LSEC clearance receptors ample possibility to remove blood-borne waste macromolecules and colloids that are incompatible with homeostasis. Not only is the sinusoidal blood flow velocity slow, the flow in individual sinusoids is characterized by temporal heterogeneity, which differs between the sinusoidal zones (MacPhee et al., 1995). The intermittence of sinusoidal blood flow varies from fast, slow, stopped, or even reversed. These different flow conditions create very different microenvironments for the liver cells, including LSECs, in zone 1 vs. zone 3. This temporal zonal flow fluctuation, which offers greatly different opportunities for LSECs to survey and bind blood-borne waste macromolecules, needs to be further studied to learn more about the regulation of the clearance activity along the hepatic sinusoid.

Several studies have been published on the expression and ligand specificity of the special LSEC endocytosis receptors, some of which are sufficiently unique to be used as LSEC specific markers at both mRNA and protein levels (Sørensen et al., 2015; Pandey et al., 2020; Sørensen and Smedsrød, 2020). When the goal is to study the LSEC role as blood clearance cells, it appears that not only anatomical aspects and the receptor expression and specificity must be included; the entire endocytic pathway in LSECs must be explored. A literature survey on this topic reveals that major cell physiological events spanning from receptor-mediated ligand internalization to lysosomal ligand processing, are more active in LSECs than in other liver cells and endothelial cells. First, the mode of endocytosis reported for ligands taken up *via* LSEC scavenger and mannose receptors is *via* the clathrin-mediated pathway (Smedsrød et al., 1988; Eskild et al., 1989; Esbach et al., 1994; Hellevik et al., 1998; Kjeken et al., 2001; Hansen et al., 2005). Soluble immune complexes are also internalized *via* clathrin-coated pits after binding to the LSEC FcγRIIb2 (Mousavi et al., 2007). This distinguishes LSECs as a unique member of the family of endothelial cells, since it is generally held that caveolae-mediated endocytosis is a characteristic of endothelial cells. LSECs express caveolin-1 (Yamazaki et al., 2013) but endocytosis *via* caveolae has not been described, and fluid-phase endocytosis is also of little importance for the scavenger function of LSECs (Kjeken et al., 2001).

Abundance of clathrin-coated pits and vesicles has been reported repeatedly in LSECs (Wisse, 1970, 1972; Kjeken et al., 2001; Falkowska-Hansen et al., 2007). These were described as "bristle-coated pits and vesicles" in the early, epoch-forming ultrastructural studies of LSECs by Wisse (1970, 1972); clathrin was first described by Pearse (1976). Morphometric analyses of rat liver showed that the density of coated pits at the plasma membrane was about twice as high in LSECs compared to Kupffer cells and hepatocytes (Kjeken et al., 2001). LSECs are highly porous cells with open fenestrae allowing direct passage of plasma proteins and lipoproteins to the subendothelial space of Disse (Wisse, 1970; Wisse et al., 1985; Fraser et al., 1995). The observation that coated pits are present both on the abluminal and adluminal aspects of the sinusoidal lining (**Figure 2**; Sørensen et al., 2012, 2015), although more abundant toward the sinusoidal lumen, indicates that endocytosis can take place on both sides of the LSEC *in vivo* allowing capture also of filtrated ligands.

**TABLE 2 |** Factors contributing to the remarkably effective blood clearance activity of LSECs.

***Factors concerning the LSECs proper:***

- Expression of dedicated waste clearing receptors with high receptor ligand affinity
- Extremely fast shuttling (recycling) time of clearance receptors between the cell surface and the early endosomal compartment
- Well-developed apparatus for intracellular trafficking and degradation of endocytosed cargo
- Content of endocytic organelles higher than in most other cell types

***Anatomical and physiological considerations:***

- Strategically located for optimal possibility to survey the blood
- Large total surface facing the blood
- Slow sinusoidal blood flow that allows optimal chance for ligands to encounter clearance receptors

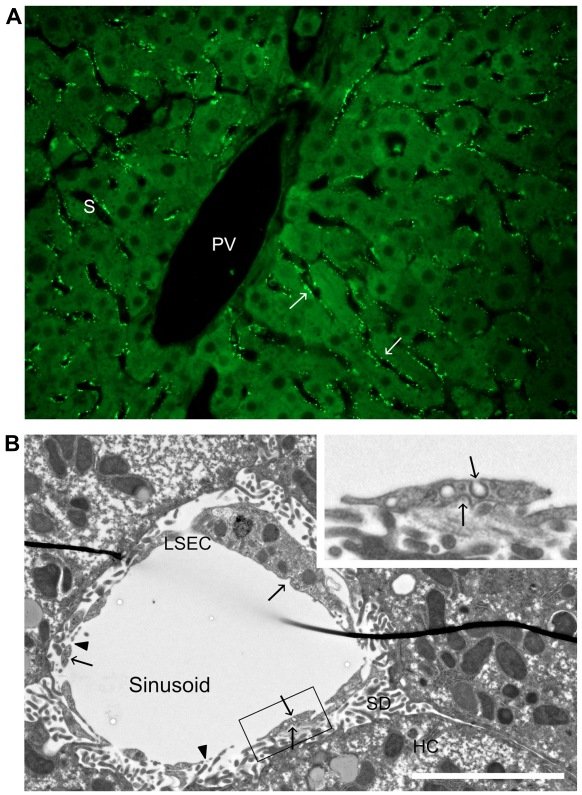


**FIGURE 2 |** Distribution of a soluble scavenger receptor ligand in the hepatic lobule, and ultrastructure of a liver sinusoid. **(A)** Uptake of FITC-FSA (formaldehyde-treated serum albumin) in mouse liver, 10 min after intravenous administration (dose 2 μg/g bodyweight). Arrows points to FITC-FSA (bright green) located along the sinusoids (S), in a pattern typical of uptake in LSECs. PV, portal vein. **(B)** Transmission electron micrograph of a rat liver sinusoid. The inserted image is a magnification of part of the LSEC in the main image. Arrows point to coated pits and arrow heads to fenestrae. LSEC, liver sinusoidal endothelial cell; SD, space of Disse; HC, hepatocyte. Scale bar 5 μm.

Receptors that internalize ligands *via* the clathrin pathway recycle to the cell surface. The half-life for internalization of receptor-ligand complexes is reported to be 17 and 10 s, for LSEC-mediated endocytosis *via* scavenger receptors (SRs; Eskild et al., 1989) and the mannose receptor (Magnusson and Berg, 1989), respectively. This is about 15–35 times as fast as internalization of ligand *via* the galactose receptor of hepatocytes [calculated from table 2 in Eskild et al. (1989)]. This very rapid receptor recycling in LSECs additionally explains the extremely effective clearance of ligands following intravenous administration. Similarly, *in vivo* the circulatory half-life of the ligands removed from blood *via* LSEC receptors are only a few minutes (**Table 1**).

Following receptor-mediated delivery of ligand to early endosomes, the ligands are transported along the endocytic pathway to the lysosomes for degradation. It is worthy of note that LSECs express very high amounts of Rab5, Rab7, clathrin, α-adaptin, β-adaptin, and rabaptin-5 (Juvet et al., 1997), which are all involved in this pathway. Comparison of the rat LSEC and Kupffer cell transcriptome and proteome further showed higher expression of genes associated with endocytic function, vesicle transport, and positive regulators of endocytosis in LSECs (Bhandari et al., 2020). This adds to the observations that LSECs are highly specialized to perform rapid endocytosis. Additional aspects supporting the notion of LSECs as specialized, professional scavenger cells, is the observations that the cells contain high amounts of lysosomes. Although the LSECs make up only 3.3% of the total liver cell volume, the cells contain impressively 45% of the organ's endocytic vesicles and 17% of the lysosomal volume (Blouin et al., 1977). Yet another factor contributing to the efficient scavenging activity of LSECs is the specific activity of several lysosomal enzymes which is higher in LSECs than in hepatocytes and Kupffer cells (Knook and Sleyster, 1980; Elvevold et al., 2008a).

In the following sections, we will focus on the major endocytosis receptors of LSECs and the ligands that they remove from the circulation. In addition, we will include information about zonation of receptor expression, species differences, and known changes in receptor expression and clearance function in disease. Finally, we include a section on comparative aspects of clearance function of LSEC-like cells in other organs, and in non-mammalian species.

## SCAVENGING RECEPTORS IN LIVER SINUSOIDAL ENDOTHELIAL CELLS

Liver sinusoidal endothelial cells express a wide range of endocytosis receptors, recently reviewed by Pandey et al. (2020) in this review series. Of these, the main receptors involved in clearance of waste molecules produced in normal turnover processes and disease include stabilin-1 and stabilin-2 (belonging to the LSEC SRs), the FcγRIIb2, and the mannose receptor (Sørensen et al., 2012, 2015).

### Liver Sinusoidal Endothelial Cell Scavenger Receptors

The term "scavenger receptor" (SR) originally described a macrophage receptor which mediates the endocytosis of a broad range of polyanionic molecules (Goldstein et al., 1979). However, this definition needs some refinement as several new SRs and their ligand specificities have been characterized since the definition was first launched. The wide range of ligands to which SRs bind, include: (i) chemically modified proteins such as acetylated and oxidized lipoproteins, maleylated bovine serum albumin (m-BSA), and formaldehyde-treated serum albumin (FSA); (ii) certain polysaccharides such as dextran sulfate; (iii) advanced glycation end-product (AGE) proteins; (iv) amino terminal procollagen propeptides; (v) four stranded, but not one or two stranded, polynucleotides such as poly-inosinic acid and poly-guanylic acid; and other ligands such as anionic lipids on the surface of damaged or apoptotic cells, endotoxin and lipoteichoic acid on pathogenic microorganisms, and crocidolite asbestos (Brown and Goldstein, 1983; Nagelkerke et al., 1983; Blomhoff et al., 1984; Krieger et al., 1993; Krieger and Herz, 1994; Melkko et al., 1994; Smedsrød et al., 1997; Yamada et al., 1998).

The physiological role of SRs is to clean up cellular debris and serve as a part of host defense, but they also play a pathophysiological role in, for example, the accumulation of oxidized LDL (oxLDL) in macrophages leading to the formation of foam cells in atherosclerosis. However, acetylated LDL (acLDL), which does not occur naturally, is a commonly used ligand in the study of SRs. Dextran sulfate is another non-endogenous polyanion used in the study of SRs. This ligand does not discriminate between SRs and mannose receptors, and is therefore regarded as a nonspecific inhibitor of receptor-mediated endocytic pathways (Jansen et al., 1991).

Liver sinusoidal endothelial cells possess significant SR activity responsible for clearing AGE-proteins (Smedsrød et al., 1997; Hansen et al., 2002b), oxLDL (Van Berkel et al., 1991), acLDL (Nagelkerke et al., 1983), hyaluronan (Eriksson et al., 1983; Smedsrød et al., 1984), chondroitin sulfate (Smedsrød et al., 1985b), amino-terminal procollagen propeptides (Smedsrød, 1988; Melkko et al., 1994), nidogen (Smedsrød et al., 1989), and FSA (Blomhoff et al., 1984) from the circulation. FSA is a well-established model ligand used to assess SR activity in LSECs (**Figure 2**), as well as determining identity and purity of LSEC preparations (McCourt et al., 1999; Sørensen et al., 2015; DeLeve and Maretti-Mira, 2017). This LSEC SR activity is independent of that attributed to the macrophage scavenger receptor (MSR1, aka SR-A1), which is also expressed in LSECs (Hansen et al., 2002a).

The SRs are a growing family [currently 12 different classes (Alquraini and El Khoury, 2020)] of structurally unrelated proteins that have a common affinity for polyanionic molecules. The nomenclature follows the classification defined in PrabhuDas et al. (2017), namely SR-A to SR-L. Of these, LSECs express receptors belonging to class SR-A, SR-B, SR-E, SR-H, SR-J, SR-K, and SR-L (reviewed in Pandey et al., 2020). Despite the expression of several SR subclasses on LSECs, the main work-horse SR on this cell type appears to be SR-H2/stabilin-2, possibly together with SR-H1/stabilin-1 (McCourt et al., 1999; Sørensen et al., 2012). It remains to be determined if the SR-E members LOX-1 and the mannose receptor on LSECs have a role in clearance of the "classical polyanionic" SR ligands. However, the LSEC mannose receptor clearly plays an important role in the clearance of circulating collagen alpha chains (Malovic et al., 2007), C-terminal propeptide of type-1 procollagen (Smedsrød et al., 1990a), tPA (Smedsrød and Einarsson, 1990), and lysosomal enzymes (Elvevold et al., 2008a) (discussed in section "The Mannose Receptor").

An important difference between human and rodent LSECs regarding SR expression is that CD36 (SCARB3) is widely expressed in human LSECs, and can thus be used as a marker for these cells in tissue sections (Strauss et al., 2017). However, comparative transcriptomic and proteomic profiling of (Sprague Dawley) rat LSECs and Kupffer cells revealed very low CD36 expression in LSECs compared to Kupffer cells (Bhandari et al., 2020), as was also reported in (Li et al., 2011).

The identification and characterization of SRs involved in blood clearance in the LSEC has been a long and winding road in part due to the belief that the LSEC hyaluronan receptor and the receptor referred to as "the LSEC scavenger receptor" were two separate entities. This issue was finally resolved in 1999 when the hyaluronan receptor and a SR on LSECs were found to be one and the same (McCourt et al., 1999), although there was already an indirect suggestion this was the case in 1986 when chondroitin sulfate (a ligand for the hyaluronan receptor) partially inhibited the uptake of a SR ligand (Eskild et al., 1986).

Hyaluronan is a widely distributed negatively charged polysaccharide, first isolated from the vitreous humor (Meyer and Palmer, 1934). It has been attributed with many biological functions such as space filling and joint lubrication, as well as other more specific effects on cell function. Fraser et al. (1981) reported the fate of hyaluronan injected into the blood of rabbits, using $^{3}$H-hyaluronan, which was labeled on acetyl groups. After 19 min, 88% of the label was detected in the liver, where it was found almost entirely in the non-parenchymal cell (NPC) fraction after Percoll fractionation of liver cells. Some radiolabel was also found in the spleen. The only metabolite detected in the blood or urine was $^{3}H_2O$, suggesting complete degradation of the polysaccharide. A subsequent whole body study of the distribution of radioactivity in mice injected intravenously with $^{14}$C-hyaluronan showed that the polysaccharide was taken up by liver, spleen, bone marrow, and lymph nodes (Fraser et al., 1983).

Eriksson et al. (1983) demonstrated that LSECs, and not Kupffer cells, were the main sites of uptake of hyaluronan by the liver. Smedsrød et al. (1984) performed further studies with primary cultures of parenchymal cells and NPCs to test their ability to bind hyaluronan (at 4°C) and internalize and degrade the ligand (at 37°C), and confirmed that LSECs (and not Kupffer cells or hepatocytes) were able to bind hyaluronan with high specificity and affinity. It was shown that the rates of hyaluronan uptake were highest in LSEC cultures, with degradation products appearing in the supernatant within 30 min of addition of $^{3}$H-hyaluronan; steady state levels of internalized $^{3}$H-hyaluronan and degradation products occurred 60–75 min into the incubation. The above results were confirmed *in vivo* with whole body autoradiography studies determining the fate of $^{3}$H-hyaluronan 10 min after injection into rats; approximately 90% of the injected radioactivity was found in the cytoplasm of LSECs, while none was found in Kupffer cells (Fraser et al., 1985).

The avidity of the endocytic hyaluronan receptor for its ligand increases with the length of the polysaccharide; the dissociation constant ranges from 1.4 μM for octasaccharides to 9 pM for hyaluronan of $6.4 \times 10^6$ Da (Laurent et al., 1986b). The smallest hyaluronan fragment that can bind is a hexasaccharide (Smedsrød et al., 1984). The rat receptor also has a threefold greater affinity for chondroitin sulfate than for hyaluronan of the same chain length, but had no affinity for heparin or heparan sulfate (Smedsrød et al., 1984; Laurent et al., 1986b). Chondroitin sulfate, as free chains and as proteoglycan and, to a lesser extent, dermatan sulfate can inhibit the uptake and binding of hyaluronan by LSECs (Smedsrød et al., 1984). Dextran sulfate, a synthetic polysaccharide not found in nature, can also inhibit the binding by LSECs (Raja et al., 1988; McGary et al., 1989).

Studies of digitonin permeabilized LSECs in suspension and culture revealed that 50–75% of the hyaluronan binding sites were intracellular (Raja et al., 1988). The hyaluronan receptors are not degraded after internalization and replaced by newly synthetized receptors, as cycloheximide, an inhibitor of protein

synthesis, had no effect on the endocytosis of hyaluronan by cultured LSECs. Instead the receptors are recycled during the continuous endocytosis of hyaluronan, proposed to be *via* a coated pit pathway (McGary et al., 1989).

The "fusion" of the LSEC hyaluronan receptor and LSEC SR activities resulted from a fortuitous discovery by McCourt et al. (1999). The LSEC hyaluronan receptor had previously been wrongly identified as ICAM-1 (McCourt and Gustafson, 1997), so a new attempt was made to purify both the LSEC hyaluronan receptor and the LSEC SR simultaneously from the same LSEC extract. The authors found instead that a Sepharose affinity column coupled with an SR ligand (amino terminal pro-peptides of type I procollagen, PINP) depleted a putative LSEC hyaluronan receptor from $^{125}$I surface labeled rat LSEC extracts, and vice versa, demonstrating that the LSEC hyaluronan receptor and an LSEC SR were one and the same. A polyclonal antibody to the affinity purified protein blocked LSEC hyaluronan uptake by 80%, and SR ligands by over 50% (McCourt et al., 1999), including AGE-proteins (Hansen et al., 2002b). Amino acid sequence data obtained from the purified rat protein (McCourt et al., 1999) lead to the cloning of the mouse form (Politz et al., 2002). In the latter study, the protein was named stabilin-2 due to its homology to stabilin-1. Both stabilin-1 and stabilin-2 are expressed on LSECs and are constitutively associated with the early endocytic pathway, irrespective of ligand binding (Hansen et al., 2005), but stabilin-1 does not bind hyaluronan (Politz et al., 2002; Prevo et al., 2004).

Stabilin-1 [STAB1, aka FEEL-1 (Tamura et al., 2003), CLEVER-1 (Irjala et al., 2003)], and stabilin-2 [STAB2, aka FEEL-2 (Tamura et al., 2003), HARE (Zhou et al., 2003)] bind a number of other ligands in common, including AGE proteins (Tamura et al., 2003; Hansen et al., 2005) and oxLDL (Li et al., 2011). However, it appears that stabilin-2 has a greater affinity for AGE proteins than stabilin-1 when expressed in CHO (Tamura et al., 2003) and HEK293 (Hansen et al., 2005) cells, while in HEK293 cells stabilin-1 has the greater affinity for mildly oxidized oxLDL and stabilin-2 has the greater affinity for heavily oxidized oxLDL (Li et al., 2011). There are other differences in stabilin-1/2 ligand binding. As mentioned above stabilin-2 (but not stabilin-1) binds hyaluronan (Politz et al., 2002; Prevo et al., 2004), while stabilin-1 (but not stabilin-2) binds SPARC (secreted protein acidic and rich in cysteine) (Kzhyshkowska et al., 2006). Interestingly, human stabilin-2 binds heparin (Harris et al., 2008), while the rat form did not (Smedsrød et al., 1984; Laurent et al., 1986b). Other ligands bound by stabilin-2 include chondroitin sulfates A, C, D, and E, dermatan sulfate and acLDL (Harris and Weigel, 2008). For a more extensive list of ligands bound by stabilin-1 and stabilin-2, see Pandey et al. (2020) in this review series.

Stabilin-2 is specifically expressed in LSECs among liver cells both in rodents and human (McCourt et al., 1999; Politz et al., 2002; Falkowski et al., 2003; Martens et al., 2006; Bhandari et al., 2020) and is a recommended LSEC marker (Geraud et al., 2010; Sørensen et al., 2015; DeLeve and Maretti-Mira, 2017). Immune histochemistry shows staining along the entire length of the hepatic sinusoid in rat (Bhandari et al., 2020), and the receptor is also widely expressed in mouse (Falkowski et al., 2003), and human sinusoids (Martens et al., 2006). In addition to liver, the presence of rat, mouse, and human stabilin-2 is demonstrated in sinusoidal endothelial cells of lymph nodes, spleen, and bone marrow (only studied in mice) (Falkowski et al., 2003; Weigel et al., 2003; Martens et al., 2006; Qian et al., 2009).

Stabilin-1 is expressed in the same organs as stabilin-2, but also in alternatively activated macrophages (M2 phenotype), and the two receptors show a similar staining pattern along the hepatic sinusoid (Politz et al., 2002; Martens et al., 2006). A recent study comparing the transcriptome and proteome of rat LSECs and Kupffer cells confirmed that both stabilin-1 and stabilin-2 were highly specific for LSECs (Bhandari et al., 2020).

## Liver Sinusoidal Endothelial Cell Scavenger Receptors in Development, Aging, and Disease

The stabilins have an interesting role in development and physiology. During embryogenesis, all endothelial cells in the developing (E13.5) rat liver express stabilin-2, but as the liver develops further, the expression becomes restricted to the sinusoidal endothelium (Yoshida et al., 2007). During aging, there is some reduction in LSEC scavenging, but the level of stabilin-1 and -2 expression in rat LSECs appears to be unchanged regardless of the age of the donor animal (Simon-Santamaria et al., 2010). Despite this age-related reduction in LSEC scavenging, considerable scavenging capacity remained in LSECs from older rats (Simon-Santamaria et al., 2010). Interestingly, in old mice there is reduced endocytosis of stabilin ligands (AGE-BSA) in centrilobular regions of the sinusoid, as observed by *in vivo* microscopy (Ito et al., 2007), and a negative shift in LSEC efficiency of degradation of the AGE proper was observed already in young adult mice compared to prepubertal mice (Svistounov et al., 2013).

In physiology, it was anticipated that the stabilins would be essential for life given their roles in waste clearance. However, stabilin-1 and stabilin-2 knockout mice were phenotypically normal, while stabilin-1/2 double knockout mice exhibited premature mortality and developed severe glomerular fibrosis, while their livers showed only mild perisinusoidal fibrosis without dysfunction (Schledzewski et al., 2011). This would suggest that while the stabilins play a vital role in maintaining health, there is considerable redundancy for their function, possibly mediated by other SRs and hyaluronan receptors. Loss of a single stabilin receptor (either stabilin-1 or stabilin-2) was, however, recently reported to significantly alter the mouse LSEC transcriptome and downregulate some genes (*Coll10*, *Lum*, and *Dec*) coding for carbohydrate binding proteins and defined as potential SRs, suggesting that loss of single receptors may influence LSEC scavenger functions to some extent (Olsavszky et al., 2021).

In certain disease states such as rheumatoid arthritis, osteoarthritis, liver cirrhosis, scleroderma, Werner syndrome, renal failure, psoriasis, and various malignancies the serum level of hyaluronan is elevated (Laurent et al., 1996). This is due either to overproduction of hyaluronan [e.g., in rheumatoid arthritis (Engström-Laurent and Hällgren, 1985), scleroderma (Engström-Laurent et al., 1985a), or psoriasis (Lundin et al., 1985)] or to impaired clearance from the blood [e.g., in liver cirrhosis (Engström-Laurent et al., 1985b)]. In the case of one

malignancy, Wilms' tumor, the overproduction of hyaluronan is so great that it causes the blood to become overly viscous (Tomasi et al., 1966; Wu et al., 1984) as well as causing defects in blood clotting (Bracey et al., 1987). This last example demonstrates the consequences of excessive levels of hyaluronan in the circulation, and therefore the importance of its removal by the LSEC stabilin-2.

## The Fc-Gamma Receptor IIb2

Liver sinusoidal endothelial cells express the endocytic FcγRIIb2 (CD32b) and are the main carriers of this receptor in liver (Mousavi et al., 2007; Ganesan et al., 2012). The FcγRIIb2 is an inhibitory FcγR and mediates endocytosis of small soluble immune complexes. These are formed in the blood circulation when either antibody or antigen is present in excess (Nydegger, 2007), and their clearance in LSECs *via* the FcγRIIb2 provides a way to remove IgG immune complexes without risk of pro-inflammatory activation (Anania et al., 2019). Larger complexes are phagocytosed by Fc receptors expressed on macrophages (Skogh et al., 1985; van der Laan-Klamer et al., 1985, 1986a,b).

The formation of immune complexes is a normal part of the immune defense against soluble antigens. However, deposition of immune complexes in tissues can trigger inflammation and contribute to pathology. Effective elimination is therefore important to preserve homeostasis. The liver is the main organ for clearance of circulating immune complexes (Arend and Mannik, 1971), and uptake of immune complexes in liver was reported more than 60 years ago (Benacerraf et al., 1959). Soluble immune complexes of human serum albumin (HSA) and anti-HSA IgG administered intravenously into rabbits were cleared in liver, with only negligible amounts recovered in lungs, kidney and spleen (Arend and Mannik, 1971). Uptake was independent of circulating complement components, as the tissue distribution was unchanged in complement depleted rabbits and assumed to take place in macrophages. Similar observations were made in mice, and doses known to induce glomerulonephritis could saturate the liver uptake system (Haakenstad and Mannik, 1974).

The first indications that LSECs, and not only macrophages, were involved in immune complex clearance came in the beginning of 1980s, when it was found that freshly isolated rat LSECs plated in serum-free media could avidly bind, but not phagocytose, sheep red blood cells coated with anti-sheep red blood cell IgG (Pulford and Souhami, 1981; Smedsrød et al., 1982). Binding was effectively inhibited by soluble complexes of heat-aggregated IgG and were not dependent on complement, suggesting the expression of FcγRs also in LSECs. Skogh et al. (1985) then reported that radiolabeled large, soluble immune complexes of dinitrophenylated (DNP)-conjugated HSA complexed by IgG distributed to Kupffer cells, whereas smaller complexes of lightly DNP-conjugated HSA complexed with IgG were taken up mainly by LSECs in rats (Skogh et al., 1985). The uptake of large immune complexes in Kupffer cells and small immune complexes in LSECs was also reported by others (van der Laan-Klamer et al., 1985, 1986a,b).

Using peroxidase-anti-peroxidase immune complexes as ligands, Muro et al. (1987, 1988) provided functional evidence of the presence of Fc receptors on Kupffer cells and LSECs both in mouse, rat, and human liver. Immune complexes were equally distributed along the sinusoidal wall, but absent in portal veins and arteries, and in central veins. Interestingly, the immune complexes were found to bind both on the luminal and abluminal aspects of the sinusoidal lining, but more frequently on the luminal side. Also, more binding was observed on LSECs than on Kupffer cells (identified by uptake of 0.5 μM latex beads), and were not present on stellate cells and hepatocytes (Muro et al., 1988). Morphometrical analyses of liver tissue short time after intravenous injection of small-sized BSA/anti-BSA IgG complexes in mice further suggested that LSECs rather than Kupffer cells were the major site for removal of these complexes from the circulation (Kosugi et al., 1992, 1993). However, a substantially higher total uptake in Kupffer cells than in LSECs has also been reported (Johansson et al., 1996). The discrepant findings may depend on the immune complex model system.

LSECs have previously been reported to carry FcγRII and III (Løvdal and Berg, 2001). However, Mousavi et al. (2007) showed by PCR that FcγRIIb2, a splice variant of FcγRIIb, was the only FcγR expressed in rat LSECs. The rat FcγRIIb2 has the same structural and regulatory functions as the mouse receptor and mediates a slow rate of endocytosis. By using an inhibitory antibody to FcγRII/CD32, the authors further proved that FcγRIIb2 was responsible for binding and uptake of soluble immune complexes in rat LSECs. FcγRIIb2 is also the only FcγR in mouse LSECs (Ganesan et al., 2012). The latter study further reported that 72% of total body FcγRIIb2 is expressed in liver, with approximately 90% of the liver receptors in LSECs and 10% in Kupffer cells. The dominating expression of this receptor in liver endothelial cells was also observed in a comprehensive single cell RNA sequencing (scRNA-seq) study which compared the transcriptomes of endothelial cells from 11 mouse tissues (Kalucka et al., 2020).

FcγRIIb has two major forms arising from mRNA splicing (Anania et al., 2019). The difference between the splice variants FcγRIIb1 and FcγRIIb2 is that the cytoplasmic tail of FcγRIIb2 contains a domain needed for accumulation in coated pits, and this domain is disrupted by a 47 amino acid insertion in RIIb1 (Miettinen et al., 1989). Therefore, only FcγRIIb2 can mediate endocytosis and internalization *via* coated pits (Miettinen et al., 1989). In addition to small soluble IgG immune complexes, ligands for the FcγRIIb2 include fibrinogen-like protein 2 (FGL2) (Liu et al., 2008) and measles virus nucleocapsid protein (Ravanel et al., 1997).

The FcγRIIb2 is partly associated with lipid rafts and uses the clathrin pathway for immune complex uptake (Miettinen et al., 1989; Mousavi et al., 2007). In LSECs, internalization *via* FcγRIIb2 is slower than *via* scavenger and mannose receptors (Løvdal et al., 2000; Mousavi et al., 2007), which was partly explained by the association of the receptor with lipid rafts. The FcγRIIb2 is a constitutively recycling receptor and traffics through lysosomal integral membrane protein-II (LIMPII) containing compartments to the LSEC plasma membrane both with and without bound ligand (Mousavi et al., 2007). The intracellular transport of immune complexes to lysosomes in LSECs is slow compared to transport of ligands that are taken up *via* scavenger and mannose receptors (Løvdal et al., 2000)

and was suggested to be partly due to repeated recycling of receptor-ligand complexes. An interesting observation was that the kinetics of endocytosis *via* SRs in LSECs was unaffected by the simultaneous uptake of immune complexes, whereas the degradation of immune complexes occurred in the same lysosomes as ligands for SRs (Løvdal et al., 2000).

The distribution of FcγRIIb2 along the hepatic sinusoid shows a different pattern in rodents and human. Immune staining of rat liver sections using the monoclonal SE-1 antibody (Ohmura et al., 1993; Tokairin et al., 2002), which specifically recognizes FcγRIIb2 in rat LSECs (March et al., 2009), showed expression along the entire length of the sinusoid (Tokairin et al., 2002; Bhandari et al., 2020). Similarly in mice, the monoclonal 2.4G2 antibody (Unkeless, 1979), reported to be specific for mouse LSECs in liver sections (Ganesan et al., 2011), stained the entire sinusoidal lining (Ganesan et al., 2012). However, in human liver, immune staining experiments showed low or absent expression of the receptor in the periportal areas (Strauss et al., 2017). This is in accordance with older functional studies showing continuous presence of uptake/binding of immune complexes (interpreted as presence of active Fc receptors) in all sinusoids of rodents, but low or absent binding/uptake close to the portal triad in human liver (Muro et al., 1987, 1988, 1993b).

### Expression and Role of the Liver Sinusoidal Endothelial Cell FcγRIIb2 in Disease

Containing the immunoreceptor tyrosine-based inhibitory motif (ITIM), FcγRIIb is the only inhibitory Fc receptor and controls many aspects of immune and inflammatory responses. Variations in the *FCGR2B* gene or lack of functional receptor are associated with susceptibility to autoimmune disease, particularly systemic lupus erythematosus (Smith and Clatworthy, 2010). FcγRIIb deficiency also increases the severity of collagen-induced arthritis (Smith and Clatworthy, 2010; increased collagen-specific IgG titres). Furthermore, since 72% of the FcγRIIb2 in mice is in the liver, and 90% of this is in LSECs, it has been speculated that inadequate expression or function of this receptor in LSECs may be a cause of serum sickness and other diseases associated with high levels of soluble immune complexes (Ganesan et al., 2012). Moreover, the high expression of FcγRIIb2 in LSECs, together with studies showing that mice lacking this receptor tend to develop systemic lupus erythematosus (Yajima et al., 2003) is additional evidence that LSECs may play a role in the aetiology of this disease.

Fc-gamma receptors are reported to be downregulated or lost in liver cirrhosis (Muro et al., 1990, 1993b) and in states of proliferation after partial hepatectomy (Muro et al., 1993a), as well as in hepatocellular carcinoma (HCC) (Geraud et al., 2013). A comprehensive single cell transcriptomics study of normal and cirrhotic mouse livers revealed zone specific alterations of LSEC receptor expression in liver cirrhosis induced by $CCl_4$ (Su et al., 2021). The study revealed three clusters of LSEC populations corresponding to hepatic zones 1–3. Expression of genes associated with capillarization such as *Cd34*, was most prominent in the pericentral zone (zone 3) in this disease model and was associated with downregulation of *Fcgr2b* (Cd32b) and other receptors. Moreover, the relative share of non-LSEC vascular endothelial cells and lymphatic endothelial cells increased in cirrhotic mice with LSECs constituting 89% of the endothelial cells in normal mouse liver, and 73% in cirrhotic livers. This may lead to decreased immune complex-clearance in LSECs, and rats with $CCl_4$-induced liver cirrhosis showed delayed clearance of immune complexes and a weakened reactivity to the ligand in the cirrhotic areas (Muro et al., 1990).

A slight reduction in CD32b expression was noted in aging rat liver but not in human liver (Maeso-Diaz et al., 2018). Interestingly, plasma levels of FGL2, a ligand for FcγRIIb and FcγRIII (Liu et al., 2008) was reported to be elevated in patients with non-alcoholic fatty liver disease (Colak et al., 2011), and in patients with liver cirrhosis and HCC (Sun et al., 2014), suggesting a link to decreased receptor expression.

CD32b, together with stabilin-1, stabilin-2, and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), were sequentially lost during tumor progression in mice with inducible HCC (AST model), as well as in human HCC patients (examined in tissue microarrays) (Geraud et al., 2013). The four LSEC markers were also lost to varying degree in the peritumoral tissue. Interestingly, loss of stabilin-2 and CD32b in the peritumoral tissue of human HCC correlated with significantly increased survival, and the authors suggested that loss of stabilin-2 and CD32b may be markers for subsets of HCC that modify the surrounding microenvironment in a different way.

## The Mannose Receptor

The mannose receptor (MRC1, CD206, or SR-E3), a type I transmembrane protein, is a member of the C-type lectin family and the SR-E family. This receptor is truly a multi-ligand clearance receptor since it has binding affinity for many different ligands in three distinct ligand binding domains. A C-type ($Ca^{2+}$-dependent) carbohydrate binding (aka C-type lectin) domain in eight copies recognizes mannose, N-acetylglucosamine, and L-fucose in the ultimate position of the glycosyl chains of glycoproteins (Ezekowitz et al., 1990; Taylor and Drickamer, 1992; Taylor et al., 1992). A second domain, characterized by a single fibronectin type II repeat, binds specifically to alpha chains of types I–IV collagen (Martinez-Pomares et al., 2006; Napper et al., 2006). A third domain, rich in cysteine, binds with high affinity to sulfated N-acetyl-galactosamine (GalNAc-4-$SO_4$) residues (Fiete et al., 1998). The two latter domains do not depend on $Ca^{2+}$ for ligand binding.

The mannose receptor is expressed on macrophage subgroups, perivascular microglia cells and several other cell types, including sinusoidal endothelial cells of liver, spleen, and lymph nodes (Linehan et al., 1999). LSECs are the main carrier of the mannose receptor in the liver of mouse, rat, and pig (Magnusson and Berg, 1989; Elvevold et al., 2004, 2008a; Linehan, 2005; Linehan et al., 2005; Malovic et al., 2007; Bhandari et al., 2020), with lower or absent expression in Kupffer cells (Magnusson and Berg, 1989; Linehan et al., 2005; Elvevold et al., 2008a; Sørensen et al., 2015). Although less explored in human liver, the mannose receptor is reported to be specifically expressed in LSECs along the sinusoids (Martens et al., 2006). Recently, a 30-gene (human) LSEC fingerprint was established based on GFP+ liver endothelial cells

from *Tie2*-GFP mice using genes with human orthologs (de Haan et al., 2020). The mannose receptor (*Mrc*1) was ranked top three of the LSEC markers measured by microarray quantification; expression in human liver was confirmed on the protein level. In contrast, scRNA-seq of human liver did not identify *MRC1* amongst the top differentially expressed genes in neither LSECs nor Kupffer cells (MacParland et al., 2018), and a recent bulk proteome and transcriptome profiling comparing rat LSECs and Kupffer cells revealed abundant expression of the mannose receptor (*Mrc1*) in both cells, with the highest expression in LSECs (Bhandari et al., 2020). From the reviewed literature we conclude that the mannose receptor is stably and highly expressed in LSECs in all species examined but that expression in liver macrophages can vary.

Differential expression and distribution patterns along the sinusoids have been described for several LSEC markers in human liver, with immunofluorescence microscopy studies establishing distinct populations of LSECs in periportal and pericentral areas (Strauss et al., 2017). Likewise, scRNA-seq of human liver revealed heterogeneity within different hepatocellular populations, with 806 out of 1,198 expressed genes in LSECs exhibiting significant zonation (Aizarani et al., 2019). However, detailed information about the mannose receptor is not highlighted in these studies. The mannose receptor is not reported to be differentially expressed along the liver sinusoid, and immune histochemical studies indicate uniform expression along sinusoids of mouse and human liver (Martens et al., 2006; Ganesan et al., 2011; Simon-Santamaria et al., 2014). Interestingly, mannose receptor scavenging activity was shown to be zonated in an IL-1β dependent way in mice (Asumendi et al., 1996). In this study, periportally located "Type I" endothelial cells significantly increased their uptake of the mannose receptor ligand ovalbumin following IL-1β treatment compared with "Type II" endothelial cells located close to the central vein.

The mannose receptor is a clearance receptor of high versatility. Several of the ligands recognized by this physiologically important receptor in LSECs is constantly released to the circulation as result of normal tissue turnover processes, and at higher rate during inflammatory episodes. They are then swiftly and silently removed from the blood by LSEC-mediated clearance. The receptor plays an important role in removing collagen fragments from the circulation. Carboxyterminal propeptides of procollagen type I, released during the formation of collagen fibers, are cleared by LSECs after binding to the mannose receptor C-type lectin domain (Smedsrød et al., 1990a). Moreover, free alpha chains of type I collagen, which are released to the circulation as a result of the ongoing connective tissue remodeling of bone and other connective tissues, were reported more than 30 years ago to be removed from the circulation in rat *via* a specific receptor in LSECs (Smedsrød et al., 1985a; Smedsrød, 1990). Receptor-ligand competition studies indicated that this receptor was distinct from other clearance receptors known at the time (Smedsrød et al., 1985a), and it was therefore named the LSEC collagen receptor. However, in 2007 the receptor was found to be identical to the mannose receptor (Malovic et al., 2007), recognizing the collagen type I alpha chains through binding to its fibronectin type II domain. The early LSEC studies further showed that alpha chains of types I, II, III, and IV collagen were internalized *via* the same receptor specificity (Smedsrød, 1989). This is compatible with results obtained from studies using mannose receptor transfection in fibroblasts, revealing that alpha chains of types I, III, and IV collagen bind to the fibronectin type II domain of the mannose receptor (Napper et al., 2006). The binding affinity of free collagen type I alpha chains to LSECs is considerably higher than the affinity to native, triple helical collagen (Smedsrød et al., 1985a; Smedsrød, 1990; Malovic et al., 2007). This makes physiological sense, since the cleavage products from the breakdown of native collagen by vertebrate collagenase, which generates the enzymatic clip that initiates extracellular degradation of native matrix collagen, readily denature at 37°C, and fall apart to free alpha chains (Sakai and Gross, 1967). The result is that free alpha chains, but not native collagen triple helices represent the blood-borne waste products of collagen. Moreover, this receptor binding preference ensures that the LSEC mannose receptor ignores the intact collagen triple helix structures in the space of Disse. It can be calculated that as much as 0.5 g collagen fragments are released daily to the circulation (Ellis, 1961; Christenson, 1997). This illustrates the importance of the LSEC mannose receptor in the clearance of collagen alpha chains from the circulation.

Another example of blood-borne molecules that are cleared by the LSEC mannose receptor is lysosomal enzymes, which contain mannose in terminal position of their glycosylation side chains. These enzymes are initially glycosylated with mannose-6-phosphate residues in the terminal position, which serves as a signal for transfer from the Golgi apparatus to the endosomal/lysosomal compartment. Once inside the lysosomes, acid phosphatase cleaves off the phosphate residues. Hence, when lysosomal enzymes leak out from cells, which takes place both under normal conditions, and at increased rates in inflammation, these molecules are effectively cleared from the circulation by binding to the LSEC mannose receptor (Hubbard et al., 1979; Isaksson et al., 1983; Elvevold et al., 2008b). There are strong indications that the very high specific activity of lysosomal enzymes in LSECs can be partly ascribed to recruitment of these enzymes from the circulation (Elvevold et al., 2008a). This hypothesis is supported by studies in mannose receptor deficient mice showing that LSECs depend on the mannose receptor for recruitment of lysosomal enzymes to maintain normal degradation capacity (Elvevold et al., 2008a).

Tissue plasminogen activator (tPA), a key hemolytic factor, is normally present in the circulation at very low levels. This is mainly due to clearance *via* the mannose receptor in LSECs and to a lesser extent by uptake in hepatocytes (Smedsrød and Einarsson, 1990). This physiologically important mechanism restricts the powerful fibrinolytic activity of tPA to act only at fibrin clots where it binds and performs its enzyme activity by activating the proenzyme plasminogen to fibrinolytic plasmin.

The N-terminal cysteine-rich domain of the mannose receptor recognizes and mediates the clearance of pituitary sulfated glycoprotein hormones, such as lutropin and thyrotropin, from the circulation. This is an important mechanism to control the level of these hormones (Simpson et al., 1999).

### Role of the Liver Sinusoidal Endothelial Cell Mannose Receptor in Inflammation and Disease

In addition to being responsible for the housekeeping clearance of waste substances, the mannose receptor on LSEC is also involved in the clearance of molecules such as lysosomal enzymes, tPA and myeloperoxidase released during the inflammatory response (Gazi and Martinez-Pomares, 2009). Thus, the mannose receptor contributes to restore homeostasis after inflammatory episodes, a function that links LSECs tightly to the resolution phase of the inflammatory response.

Through its recognition and binding of exogenous molecules such as virus, bacteria and fungi by the C-type lectin domains, the mannose receptor is considered to be an important pattern recognition receptor (PRR) involved in host defense (Stahl and Ezekowitz, 1998). Interestingly, mannose receptor deficiency did not translate into increased susceptibility to infection with *Candida albicans*, *Pneumocystis carinii*, or *Leishmania* spp. in mice (Lee et al., 2003; Swain et al., 2003; Akilov et al., 2007), but variations in the mannose receptor gene (*MRC1*) may be associated with increased susceptibility to chronic inflammatory diseases such as asthma and sarcoidosis in humans (Hattori et al., 2009, 2010). In liver disease, the soluble mannose receptor is used as a macrophage activation marker to predict disease severity and prognosis in conditions such as alcoholic liver disease, primary biliary cholangitis, and Hepatitis B (Sandahl et al., 2017; Li et al., 2019; Bossen et al., 2020).

Due to their anatomical location, LSECs are the first cell type to encounter blood-borne antigens reaching the liver. Hence, it is not surprising that these cells have important innate and adaptive immunological functions (Shetty et al., 2018). In addition to the silent removal of waste molecules, endocytosis of ligands by some SRs, including the mannose receptor, may promote potent pro-inflammatory and anti-inflammatory signaling (Canton et al., 2013). Several receptors highly expressed by LSECs have been shown to interact with different viruses (Lin et al., 2003; Marzi et al., 2004; Gramberg et al., 2005; Lai et al., 2006; Li Y. et al., 2009) and the mannose receptor may mediate dengue virus infection of human macrophages (Miller et al., 2008). Many viruses are highly mannosylated (Zhang et al., 2004), which makes them a likely ligand for the mannose receptor; however, the contribution of the mannose receptor to viral uptake in LSEC is unknown. LSECs can also cross-present antigens to CD8+ T cells by the help of the mannose receptor which takes up, processes and transfers antigen to MHC class I molecules (Limmer et al., 2000; Burgdorf et al., 2007), a process that has been shown to promote CD8+ T cell tolerance in mice (Schurich et al., 2009).

## Other C-Type Lectins and Receptors With Suggested Roles in Liver Sinusoidal Endothelial Cell Blood Clearance

Besides the mannose receptor, LSECs express several other receptors in the c-type lectin family, including L-SIGN (DC-SIGNR and CLEC4M), and LSECtin (CLEC4G) (Bhandari et al., 2020).

In a study comparing the sequenced mRNA transcriptome and proteome of LSECs and Kupffer cells from Sprague Dawley rats, L-SIGN was highly expressed in LSECs only, and low in Kupffer cells (Bhandari et al., 2020). L-SIGN is also strongly and constitutively expressed in human (Pohlmann et al., 2001) and mouse LSECs and can be upregulated in response to treatment with cytokines (Lai et al., 2006). The functional role of the receptor on LSECs is however, not well known, but L-SIGN on other endothelial cells can bind viruses such as HCV (Gardner et al., 2003) and HIV (Pohlmann et al., 2001). Recently, human L-SIGN was shown to act as a receptor for SARS-CoV-2 (Kondo et al., 2021) and the hypothesis was presented that L-SIGN mediated SARS-CoV-2 infection in LSECs, and subsequent activation of the sinusoidal endothelium contributes to COVID-19-associated coagulopathy in patients.

Liver and lymph node sinusoidal endothelial cell C-type lectin is related to L-SIGN and is expressed predominantly by sinusoidal endothelial cells of human liver and lymph nodes (Liu et al., 2004). In a study establishing a 30-gene (human) LSEC signature (de Haan et al., 2020), LSECtin/CLEC4G was ranked as the most highly expressed LSEC marker protein in mouse liver tissue. Expression was also high in rat LSECs compared to Kupffer cells (Bhandari et al., 2020).

High mRNA expression of LSECtin/*CLEC4G*, as well as L-SIGN/*CLEC4M*, has also been shown in human LSECs by single cell sequencing of liver cells (Aizarani et al., 2019). *CLEC4G* was further found on the list of the top 20 most differentially expressed genes in the human liver endothelial cell cluster hypothesized to correspond to "Type-2" LSECs (midzonal and pericentral area), while not appearing on the list of differentially expressed genes in the endothelial cluster corresponding to "Type-1" LSEC (periportal area) (MacParland et al., 2018), indicating a similar zonated pattern as reported for *LYVE1* (Strauss et al., 2017). The LSECtin receptor binds to mannose, N-acetylglucosamine (GlcNAc) and fucose, and has been reported to act as a receptor for different viruses such as the Japanese encephalitis virus (Shimojima et al., 2014), filovirus (Ebola), SARS Coronavirus (Gramberg et al., 2005), Lassa virus (Shimojima et al., 2012) and the lymphocytic choriomeningitis virus glycoprotein (Shimojima and Kawaoka, 2012). The contribution by LSECtin in viral uptake is not well known, but the receptor is potentially involved in the regulation of immune responses toward HCV through interaction with L-SIGN (Li Y. et al., 2009). Although possibly mediating viral uptake, the role of LSECtin in LSEC endocytosis is so far unknown.

Lymphatic vessel endothelial hyaluronan receptor (LYVE-1) is a hyaluronan receptor initially believed to be predominantly located in lymphatic endothelial cells (Banerji et al., 1999; reviewed in Jackson, 2004). Constitutive expression of LYVE-1 is also found in LSECs (Mouta Carreira et al., 2001) and sinusoidal endothelia of human lymph nodes and spleen (Banerji et al., 1999), as well as in vascular endothelial cells of murine lung, adrenal gland, and heart (Zheng et al., 2016) and subsets of tissue macrophages (Schledzewski et al., 2006). Immune labeling of tissue sections show that the distribution of the receptor in human liver is zonated along the sinusoids with LSECs in the periportal area (hepatic zone 1) being negative or low for LYVE-1 while LSECs in midzonal and pericentral areas (hepatic zones 2 and 3) have a high expression of LYVE-1 (Strauss et al., 2017).

Differential expression of *LYVE1* in distinct populations of liver endothelial cells was also confirmed by scRNA-seq of human liver cells (MacParland et al., 2018). A zonated expression pattern of LYVE-1 is also reported in mouse liver with the strongest signal observed in the midzonal sinusoids (Mouta Carreira et al., 2001).

Putative functions of LYVE-1 are hyaluronan clearance from the lymph (Prevo et al., 2001) and regulation of leukocyte adhesion and migration within the lymphatic circulation (reviewed in Jackson, 2004). Stabilin-2 is considered the major endocytic receptor for hyaluronan in LSECs (McCourt et al., 1999; Zhou et al., 2000; Harris and Baker, 2020), leaving the relative contribution of LYVE-1 in this process to be unknown. The contribution of LYVE-1 to endocytosis of other endogenous ligands, as well as elimination of foreign particles circulating in the blood, is not fully explored, but mRNA expression of *Lyve1* in murine liver and lung was increased within 4–8 h after LPS-stimulation (Zheng et al., 2016). Endocytosis of 20 nm latex particles by endothelial cells was also increased following LPS-stimulation, but only observed in the lung. LYVE-1 is further suggested to have a role in wound healing and tumor formation (Schledzewski et al., 2006).

The expression of some of these receptors has been reported to be affected by pathological conditions, with LYVE-1 (along with stabilin-1, stabilin-2, and FcγRIIb) being downregulated in human liver cancer (HCC) and cirrhosis (Mouta Carreira et al., 2001; Geraud et al., 2013), and LSECtin being downregulated in HCC (Aizarani et al., 2019).

## LIVER SINUSOIDAL ENDOTHELIAL CELL SUBPOPULATIONS AND HETEROGENEITY

An increasing number of studies show spatial heterogeneity of hepatic cells (including hepatocytes, LSECs, hepatic stellate cells, and Kupffer cells) along the porto-central axis (Strauss et al., 2017; Felmlee et al., 2018; Aizarani et al., 2019; Ben-Moshe and Itzkovitz, 2019; Blériot and Ginhoux, 2019; Ma et al., 2020; Koch et al., 2021; Payen et al., 2021). Historically, Wisse et al. (1983) reported an increase in the frequency of fenestrae in LSECs from the portal tract toward the central vein (Wisse et al., 1983). Continual studies on this aspect during the 1990s expanded our knowledge about the differential LSEC response along the sinusoids against various stimuli, substantiating the notion of some functional heterogeneity along the sinusoid (Scoazec et al., 1994; Asumendi et al., 1996; Dini and Carla, 1998). Recently, two LSEC subtypes were reported to exist along the human hepatic sinusoid, based on immune histochemistry of normal human liver, with low or absent expression of CD32 and LYVE-1 periportally (Strauss et al., 2017). The application of single-cell sequencing protocols in addition to conventional methods allows information about tissue complexities (cellular compositions) and cellular heterogeneity, the phenotype of a rare cell population, or the disease-associated cellular phenotype. Recently, several scRNA-seq studies have unraveled the complexity of the liver tissue and comprehensively characterized the hepatic cell types at the molecular level. ScRNA-seq studies have undoubtedly strengthened the evidence and validated the complex labor division among various hepatic cell types, heralding the tremendous spatial heterogeneity and complexity within liver lobules (Halpern et al., 2017, 2018; MacParland et al., 2018; Aizarani et al., 2019; Ben-Moshe et al., 2019; Ramachandran et al., 2019). Studies suggest that more than 50% of the expressed genes within hepatocytes, as well as in LSECs, show zonation (Halpern et al., 2018; Ben-Moshe et al., 2019). So far, few of these gene expressions have been validated with complementary techniques at single cell levels, and functional studies will be needed to understand how differences in gene expression along the sinusoids may affect LSEC scavenger functions.

## SCAVENGER ENDOTHELIAL CELLS IN OTHER VASCULAR BEDS

The important clearance function of LSECs is well documented (Sørensen et al., 2012). It is noteworthy, however, that specialized endothelial cells exhibiting LSEC-like clearance activity are present also in some organs other than liver. The early vital stain investigators observed accumulation of stains like lithium carmine in several organs in addition to the hepatic RES; ample uptake was reported in the "reticuloendothelium" of spleen, lymph nodes, bone marrow, adrenal cortex, and pituitary anterior gland (Kiyono, 1914; Aschoff, 1924). Although the investigators at the time had no means to accurately identify the RES cells of these organs, the conclusion nearly a century later that intravenously administered lithium carmine is cleared mainly by the LSECs in liver (Kawai et al., 1998), indicates that the cells in other organs that were noted to take up this vital stain, were LSEC-like SECs, in addition to macrophages.

Studies on clearance of physiological waste macromolecules in extra-hepatic RES organs are scarce. In mice, specialized SECs of the bone marrow, which line the sinusoids of this organ, express functional stabilin-1 and stabilin-2, enabling these cells to take up ligands (FSA, AGE-products) that are also avidly taken up *via* these receptors by LSECs (Qian et al., 2009). Likewise, alpha chains of type I collagen, a physiological ligand for the LSEC mannose receptor, were also cleared by the bone marrow SECs, suggesting the presence of both stabilin-1 and -2 and mannose receptors in these cells (Qian et al., 2009). In pig, uptake of FSA and the mannose receptor ligand α-mannosidase were observed in lung endothelium, in addition to uptake in LSECs (Nedredal et al., 2003).

Lymph nodes and spleen, two other extrahepatic organs suggested by the early vital stain scientists as RES members, express several of the same signature clearance receptors as those found in LSECs (Martens et al., 2006). Human lymph node and spleen tissue analyzed by gene profiling and immune histochemistry here demonstrated the presence of stabilin-1, stabilin-2, LYVE-1, and the mannose receptor in sinusoidal endothelial cells of these organs.

Choriocapillaris endothelial cells (CCEs) have recently been implicated as SECs, employing stabilin-2 to clear waste molecules generated in the metabolically active retina (Li R. et al., 2009). It

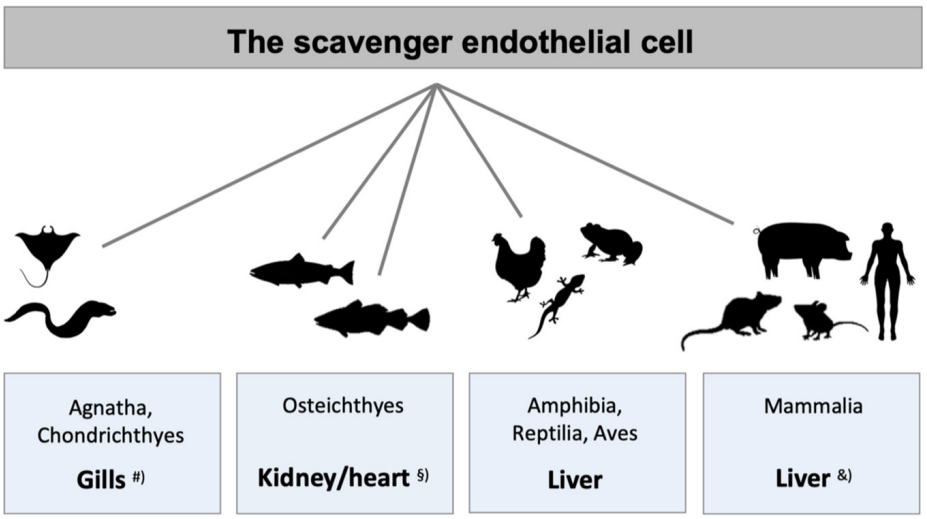


**FIGURE 3 |** Species differences in the localization of main populations of scavenger endothelial cells (SECs). The figure illustrates the organs that harbor the main populations of specialized SECs in different vertebrate classes. #SECs are localized in special gill arteries in hagfish, lamprey (both Agnatha), and ray (Chondrichthyes) (Seternes et al., 2002). § In adult bony fish (Osteichthyes) SECs constitute the endothelium of the venous sinusoids in the kidney hematopoietic tissue in crucian carp (Seternes et al., 2002) and salmonid fish (Dannevig et al., 1990, 1994; Smedsrød et al., 1993; Seternes et al., 2002), and the atrial and ventricular endocardium in Atlantic cod (Smedsrød et al., 1995; Sørensen et al., 1997, 1998, 2001; Seternes et al., 2001a, 2002). In all higher vertebrate classes LSECs represent the major SEC population, studied in frog (Seternes et al., 2002), lizard (Seternes et al., 2002), chicken (Seternes et al., 2002), rodents (Smedsrød et al., 1990b; Seternes et al., 2002; Sørensen et al., 2015), and pig (Nedredal et al., 2003; Elvevold et al., 2004). &In addition to the central scavenger function of LSECs in mammals, studies in rabbit and rodents also show scavenging function of the sinusoidal endothelium in spleen, bone marrow, and lymph nodes (Fraser et al., 1983; Qian et al., 2009; Simon-Santamaria et al., 2014), and in pig, scavenging activity is reported in lung endothelium, in addition to LSECs (Nedredal et al., 2003).

was proposed that CCEs play a significant role in the clearance of AGE products, that – if allowed to accumulate – may contribute to the generation of age-related macular degeneration. The study was done with cells from bovine eyes, and studies in human CCEs is needed to follow up the hypothesis.

## Phylogenetic Aspects – Scavenger Endothelia in Other Vertebrate Classes

The findings by the early vital stain scientists suggested that not only mammals, but also species belonging to the other classes of the vertebrate kingdom, were equipped with a RES that accumulated vital dyes (Kiyono, 1914). However, animal species of phylogenetically older vertebrates displayed a distinct, yet different RES distribution than in the land-based vertebrates. Hypothesizing that this distribution might reflect the distribution of SECs, a study was carried out to investigate if ligands reported to be taken up by LSEC clearance receptors in mammals could be used to determine the distribution of RES in vertebrate classes other than mammals (Seternes et al., 2002). The result of this screening study, summed up in **Figure 3**, revealed that ligands for the mammalian signature LSEC clearance receptors stabilin-2 and the mannose receptor, were indeed cleared from the circulation in the RES organs reported by the early vital stain scientists. In addition, the finding that particles large enough to be cleared exclusively by phagocytosis accumulated in macrophages, revealed the presence of a pan-vertebrate dual cell principle of blood clearance, with particles >200 nm taken up mainly in macrophages, while macromolecules and colloids <200 nm were cleared mainly by uptake in SECs (Seternes et al., 2002). The ligand distribution screening was performed by recording the anatomical site of ligand uptake following intravenous administration of selected (fluorescence- or radiolabeled) soluble SR and mannose receptor ligands. It is noteworthy that the endocardially located SECs of Atlantic cod (*Gadus morhua*) responsible for the blood clearance of the tested ligands in this species, express stabilin-2, as shown by western blot analysis revealing that lysates from purified cod endocardial endothelial cells, and pig and rat LSECs all reacted with an antibody to whole rat stabilin-2 (Sørensen et al., 2012).

Moreover, recent studies in embryonic zebrafish (*Danio rerio*) showed ample uptake of hyaluronan in SECs located in the caudal vein and vein plexus. This uptake was completely abolished in mutants lacking functional stabilin-2 (Campbell et al., 2018). These findings in the Atlantic cod and zebrafish show that stabilin-2 is well conserved over the considerable phylogenetic time span from bony fishes to mammals. A similarly high degree of phylogenetic conservation is also suggested for the mannose receptor, which is present not only in mammals. It has also been cloned and characterized in the zebrafish (Wong et al., 2009; Zheng et al., 2015). The expression of mannose receptor mRNA was much higher in kidney than in other organs of the zebra fish. Although the role of the zebrafish mannose receptor in the clearance of the same physiological waste molecules as in mammals has not yet been confirmed, the deduced amino acid sequences shared highly conserved structures with the corresponding mammalian receptor and contains a cysteine-rich domain, a single fibronectin type II

domain, and eight C-type lectin domains. This strongly indicates that this receptor in the zebrafish serves the same blood clearance function as in mammals.

Stabilin-1 is also expressed in zebrafish and is required for clearance of small (6–30 nm) anionic nanoparticles from the circulation, whereas a combined contribution of stabilin-1 and stabilin-2 is required for clearance of larger (approximately 100 nm) anionic nanoparticles. This finding represents significant information about the influence of the size of anionic nanoparticles for targeting the mammalian LSECs (Arias-Alpizar et al., 2021).

A recent study in 5-day-old zebrafish embryos showed that brain lymphatic endothelial cells (BLECs) play an important role as SECs in the brain, taking up waste substances such as proteins, polysaccharides and virus particles (Huisman et al., 2021). Interestingly, it was found that BLECs and microglia (brain macrophages) work side by side to remove extracellular components from the brain, thus maintaining homeostasis in the brain meninges. In this collaborative function, BLECs, like LSECs and other vertebrate SECs, are particularly active in the clearance of macromolecules and nano particles up to a certain size, whereas the microglia are more active in the uptake of larger material, e.g., bacteria. This collaborating arrangement of the two clearance cells of the brain is another striking example of the vertebrate dual cell principle of waste clearance (Sørensen et al., 2012).

At variance from the observation in mammals that hyaluronan and other waste macromolecules administered subcutaneously or intramuscularly are largely taken up in SECs of local lymph nodes, with only low amounts being cleared by LSECs, radiolabeled hyaluronan injected subcutaneously in the Atlantic cod was taken up mainly in the endocardial SECs (Sørensen et al., 1997). The lack of lymph nodes in fish explains this observation, demonstrating the importance of blood clearance of waste macromolecules in the main SEC organs of these species. Following development of a method for isolation and culture of primary cod endocardial endothelial cells (representing cod SECs) (Koren et al., 1997), studies were carried out *in vitro* to explore in more detail the effective mechanism of the uptake of physiological waste macromolecules in these cells (Koren et al., 1997; Sørensen et al., 1998, 2001; Seternes et al., 2001b). Those studies revealed that the cod SECs endocytose ligands for the scavenger and mannose receptors in the same way as has been demonstrated for mammalian LSECs. Receptor-mediated endocytosis and degradation was responsible for rapid and high-capacity uptake of the physiological molecules hyaluronan (Sørensen et al., 1997), chondroitin sulfate (Seternes et al., 2001b), lysosomal enzymes (Sørensen et al., 2001), N-terminal propeptide of type I procollagen (Sørensen et al., 1998), and collagen alpha chains (Smedsrød et al., 1995; Koren et al., 1997).

## CONCLUDING REMARKS

As the result of normal metabolic processes, large amounts of macromolecules from various tissues must be swiftly and silently removed to clean the blood and maintain homeostasis. The LSECs exhibit a remarkably efficient blood clearance capability. This is due largely to their extremely rapid and high-capacity endocytosis, mediated by receptors specifically recognizing a variety of different waste macromolecules. Moreover, the LSECs, lining the hepatic sinusoids, are strategically located for optimal survey of the blood. Equipped with endocytic pattern-recognition receptors that display multi-ligand binding domains, these cells clear a plethora of different types of waste molecules, many of which are DAMPs and PAMPs with the potential to activate immune cells if allowed to circulate. Hence, the waste clearance activity of LSECs represents a silent removal of molecules, maintaining homeostasis.

Our knowledge about the clearance activity of LSECs in various pathophysiological conditions are rudimentary. Questions that need to be answered include establishing how liver is affected by changes in the LSEC scavenger function in various pathophysiological conditions. Moreover, development of the new generation of pharmaceuticals including macromolecular and nanosized compounds are seriously hampered due to undesired clearance of these compounds in LSECs. This is still a major challenge that needs to be solved.

Studies in various mammalian tissues have revealed the presence of SECs with striking functional similarity to the LSECs. Animal species from all vertebrate classes employ SECs to clear waste macromolecules from the circulation, in the same way as LSECs of mammals. However, it is noteworthy that phylogenetically old vertebrate classes (jawless, cartilage, and bony fishes) carry their SECs in organs other than liver. Apart from this difference, the functional similarities of SECs from all vertebrates are prominent, revealing a remarkably well conserved pan-vertebrate waste clearance system that has been well conserved over a considerable phylogenetic time span.

## AUTHOR CONTRIBUTIONS

All authors substantially contributed to the design and writing of the review. AL: figures. KS: images. KS and BS: editing. All authors approved the final version.

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# Gene Signatures Detect Damaged Liver Sinusoidal Endothelial Cells in Chronic Liver Diseases

*Stefaan Verhulst*†, Elise Anne van Os*†, Vincent De Smet, Nathalie Eysackers, Inge Mannaerts and Leo A. van Grunsven**

*Liver Cell Biology Research Group, Vrije Universiteit Brussel, Brussel, Belgium*

Liver sinusoidal endothelial cells have a gatekeeper function in liver homeostasis by permitting substrates from the bloodstream into the space of Disse and regulating hepatic stellate cell activation status. Maintenance of LSEC's highly specialized phenotype is crucial for liver homeostasis. During liver fibrosis and cirrhosis, LSEC phenotype and functions are lost by processes known as capillarization and LSEC dysfunction. LSEC capillarization can be demonstrated by the loss of fenestrae (cytoplasmic pores) and the manifestation of a basement membrane. Currently, no protein or genetic markers can clearly distinguish healthy from damaged LSECs in acute or chronic liver disease. Single cell (sc)RNA sequencing efforts have identified several LSEC populations in mouse models for liver disease and in human cirrhotic livers. Still, there are no clearly defined genesets that can identify LSECs or dysfunctional LSEC populations in transcriptome data. Here, we developed genesets that are enriched in healthy and damaged LSECs which correlated very strongly with healthy and early stage- vs. advanced human liver diseases. A damaged LSEC signature comprised of *Fabp4/5* and *Vwf/a1* was established which could efficiently identify damaged endothelial cells in single cell RNAseq data sets. In LSECs from an acute $CCl_4$ liver injury mouse model, *Fabp4/5* and *Vwf/a1* expression is induced within 1–3 days while in cirrhotic human livers these 4 genes are highly enriched in damaged LSECs. In conclusion, our newly developed gene signature of damaged LSECs can be applicable to a wide range of liver disease etiologies, implicating a common transcriptional alteration mechanism in LSEC damage.

**Keywords: LSEC, acute liver injury, single cell RNA sequencing (scRNAseq), primary cells, NAFLD (non-alcoholic fatty liver disease), non-alcoholic steatohepatitis (NASH)**

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***Edited by:***
*Jinhang Gao,*
*Sichuan University, China*

***Reviewed by:***
*Kunimaro Furuta,*
*Mayo Clinic, United States*
*Jianwen Lu,*
*The First Affiliated Hospital of Xi'an Jiaotong University, China*

***Correspondence:**
*Stefaan Verhulst*
*stefaan.verhulst@vub.be*
*Leo A. van Grunsven*
*leo.van.grunsven@vub.be*

†*These authors share first authorship*

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## INTRODUCTION

Liver sinusoidal endothelial cells (LSECs) comprise about 15–20% of the total number of liver cells and line the sinusoidal lumen of the liver sinusoids. LSECs are highly specialized endothelial cells characterized by fenestrae and lack of a basement membrane (1) making these cells the most permeable cells in the mammalian body (2). LSEC permeability is important for liver function as it permits plasma, solutes, and small substrates such as albumin (3) and insulin (4) to diffuse from the blood toward the parenchymal cells. Besides working as a filter and first barrier of the liver, these cells have other functions such as the production of coagulation factor VIII (5), antigen presentation (6, 7), leukocyte recruitment and endocytosis of virus particles (8), oxidized

LDL (9) and immunocomplexes by the abundant expression of multiple scavenger receptors (10). Expression of specific scavenger receptors and other characteristic proteins can vary across the liver acinus (11, 12).

Maintenance of the specialized LSEC phenotype is essential for liver homeostasis (13). During liver injury LSECs can become dysfunctional, characterized by the loss of fenestrae and the appearance of a basement membrane, also known as capillarization (4, 14–16). LSECs can contribute to liver regeneration and healing by orchestrating an angiocrine response that can lead to a pro-regenerative response after an acute injury or to a maladaptive pro-fibrotic response after chronic injury, which in turn leads to fibrosis (17). Moreover, LSECs are described to have a gatekeeper function in liver fibrosis as differentiated LSECs promote HSC quiescence, and restoration of LSEC differentiation can prevent fibrosis progression and accelerate fibrosis regression (18). Although it is known that LSECs play an important role in the response to acute and chronic liver injury, research on the transcriptomic and phenotypic change of LSECs during acute and chronic injury is still limited. In addition, identification of LSECs using genetic/protein markers is still quite controversial (19) as there is no unique marker that characterizes LSECs (11, 13) apart from fenestrae and the absence of a basement membrane. The identification of damaged LSECs in an acute or chronic setting is even more challenging. Recently, specific markers for LSECs in healthy livers have been described, such as CD32b (20), CLEC4G (21), LYVE1 (22), STAB2 (23) in addition to the more controversial endothelial cell (EC) markers VWF and CD31 which are upregulated in LSECs during disease (13, 19). However, currently electron microscopy is still the golden standard for identification of damaged LSECs (loss of fenestrae). The recent use of single-cell transcriptomics (scRNAseq), performed on both healthy and diseased human and mouse livers, has identified several heterogeneous hepatic cell populations, including LSECs (12, 21, 24–26). These publicly available data sets present bioinformatic opportunities to define LSEC populations more efficiently in both healthy and diseased livers, independent of the etiology or background.

In this study, we developed healthy- and damaged LSEC enriched gene sets and signatures using healthy and cirrhotic human liver scRNAseq data and newly generated datasets from healthy and acutely injured mouse livers. These LSEC genesets and signatures can identify the health status of LSECs in mouse and human bulk transcriptome or scRNAseq data from chronic or acute liver diseases. Using these gene sets, we demonstrate that LSECs are dysfunctional in multiple end-stage liver diseases and that LSECs are quickly damaged upon an acute liver injury. These results highlight the important role of LSECs in liver pathophysiology.

# MATERIALS AND METHODS

## Animals

All methods and protocols were carried out according to the approved guidelines of the Vrije Universiteit Brussel (VUB, Belgium) and according to European Guidelines for the Care and Use of Laboratory Animals. Animal experiment protocols were approved by the Ethical Committee of Animal Experimentation of the Vrije Universiteit Brussel (VUB, Belgium, 14-212-4). BalbC mice aged 11–14 weeks were housed in a controlled environment in conventional cages and were allowed food and water *ad libitum*. Acute liver injury in BalbC mice was induced by a single intraperitoneal injection with 15 μl carbon tetrachloride ($CCl_4$, 87031, Sigma-Aldrich, St. Louis, MO, USA) and 85 μl mineral oil (Sigma-Aldrich, St. Louis, MO, USA) per 30 g bodyweight. Blood, total liver and cells were collected from healthy mice and after 1, 3 and 7 days of $CCl_4$ administration. Mice were anesthetized using 100 μL Dolethal® (Vetoquinol, France). Analysis of alanine aminotransferase (ALT) was performed using a SPOTCHEM EZ SP-4430 (A.Menarini Diagnostics, The Netherlands). At the start and end of the experiment mice were weighted. Daily observation of the mice showed only a mild effect on animal welfare.

## LSEC Isolation From Mice

Non-parenchymal cells (NPCs) were retrieved as previously described (27). Red blood cell lysis (Miltenyi Biotec, Germany) was performed, and NPCs were washed with PBS + 0.1% Bovine Serum Albumin (BSA). NPCs were resuspended in BPE buffer (PBS with 5% BSA and 2 mM EDTA) with 1 ul anti-mouse Fc block™ (Becton-Dickinson, Belgium) reagent added per $10^7$ cells for 10 min at 4°C. Cells were washed and incubated in 600 μL PBS+0.1% BSA with 5 μl CD32-PE (ab30357, Abcam, UK), 2 μL CD45-FITC (11-0451-85, eBioscience, USA) and 10 μl F4/80 Alexa-647 per $10^7$ cells (MF48021, Life Technologies) for 15 min at 4°C. After incubation with the antibodies, cells were washed and resuspended in a buffer solution without calcium and supplemented with DNase I (3:1, 10104159001, Roche, Switzerland) before cell isolation using FACS (FACS Aria IIu, BD Biosciences, Belgium). FACS was used to sort viable cells (negative selection based on propidium iodide) and LSECs were selected and sorted based on a positive signal for CD32 (27–29) and a negative signal for UV, F4/80, CD45. CD32b is expressed in all LSECs across the liver sinusoid (**Supplementary Figure 1A**) (30). Potential doublets with HSCs, KCs, and immune cells were excluded (cfr. **Supplementary Figure 1B**. Utmost right FACS plot with circled LSEC population). Stainings were performed on cytospins after isolation and showed a high purity (95%) of LSECs using this sorting strategy (**Supplementary Figure 1C**).

## RNA Preparation and Sequencing

Total RNA was extracted from FACS-isolated LSECs using ReliaPrep RNA Cell Miniprep System (Z6012, Promega, USA), RNA concentrations and quality measurements were performed using a Bioanalyzer 6000. Preparation of samples and sequencing, using Clontech SMARTseq v4 kit (R400752, Takara, Japan) and NovaSeq S2 (2 × 100 bp), was performed by the BRIGHTcore of the Vrije Universiteit Brussel. Single-end sequencing was run on Illumina NextSeq 500 High.

## Immunofluorescence

Mouse liver tissues were fixed with formalin for 48 h at 4°C. Liver tissues were stored in 70% EtOH and were used for sectioning (Leica, The Netherlands) of 100 μm liver sections

in 4% UltraPure™ Low Melting Point Agarose (Invitrogen, USA) using a vibratome (Leica, The Netherlands). Sections were kept in 70% EtOH until usage. Upon usage sections were rehydrated in 50% EtOH for 10 min and rinsed for 10 min with PBS. For permeabilization, sections were incubated with PBS + 0.2% Triton for 20 min at room temperature. After permeabilization, sections were washed two times with PBS and blocked with 3% BSA-PBS for 2 h at room temperature. Sections were incubated overnight at room temperature with the following primary antibodies; Lyve1 (2 μg/mL, AF2125, R&D systems, Canada), Ki67 (0.5 μg/mL, 14-5698-82, Thermofisher, USA) and CD32b (10 mg/mL, AF2125, R&D systems, Canada). PHEM buffer (10 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl2*6H2O) was used for CD32b staining instead of PBS in all steps. Vibratome sections were washed three times with PBS for 10 min and were incubated for 1 h with the following secondary antibodies (1:200); Donkey-anti-goat Alexa488 (A11055, Thermofisher, USA) and Donkey-anti-rat Alexa 647 (ab150155, Abcam, UK). Sections were washed three times with PBS, incubated for 10 min with 70% EtOH and then incubated with 1% Sudan Black (199664, Sigma-Aldrich, Belgium) in 70% EtOH. Sections were rinsed with PBS and mounted with Mowiol (9002-89-5, Sigma-Aldrich, Belgium) with DAPI (D9564, 10 μg/mL, Sigma-Aldrich, Belgium) and visualized by EVOS M7000 (Thermofischer, USA) and Zeiss Axioscan (Zeiss, Germany). Quantification was performed with HALO 3.1 image analysis platform (Indica labs Inc., USA).

## Immunohistochemistry

Liver tissues were embedded in paraffin, sliced in 5 μm sections and deparaffinized with Xylene. For H&E stainings sections were rehydrated, washed with PBS and counterstained with Harris Hematoxylin (1:10 Roth, Newport Beach, CA, USA) before being rinsed with acid water followed by 10 min wash with tap water. Sections were incubated with eosin for 5 min, shortly rinsed, dehydrated and mounted with DPX mounting medium (Sigma-Aldrich, Belgium). For Collagen 4 staining, sections were rehydrated, washed with PBS-0.05%Tween (PBST) and endogenous peroxidase was quenched with 3% $H_2O_2$ in methanol. Samples were washed three times with PBST for 5 min and incubated with 2% BSA-PBS for 1 h at room temperature. Col4 antibody (2 μg/ml, ab6586, Abcam, UK) was dissolved in 1% BSA-PBS and incubated overnight at 4°C. Sections were washed and incubated with Dako EnVision+ System- HRP Labeled Poly (K4003, Dako, Denmark) for 30 min at room temperature. Sections were washed with PBST, incubated with DAB substrate for 3 min at room temperature. Finally, samples were rinsed, counterstained with Harris Hematoxylin (1:10) and mounted with DPX mounting medium (Sigma-Aldrich, Belgium) and imaged visualized with Leica Aperio CS2 (Leica, The Netherlands). Quantification was performed with Orbit image analysis (31).

## Bioinformatics

### scRNAseq Analysis

Raw counts of scRNAseq data from healthy and diseased livers of Ramachandran et al. (GSE136103) (25), MacParland et al. (GSE115469) (24), Aizarani (GSE124395) (21), Xiong et al. (GSE129516) (26), and Terkelsen et al. (GSE145086) (32) was downloaded from GEO-NCBI database and imported into RStudio (https://www.rstudio.com). General scRNAseq analysis for quality controls, normalization, clustering and multidimensional reduction was performed using the default pipeline of R package Seurat (33). Identification of different cell clusters was performed using markers from the original publications and visualized in a UMAP plot.

### Differential Expressed Genes in scRNAseq Data

Genes differentially expressed between two populations were identified using the *findmarker* function within R package Seurat with fold changes larger than 2.

### Downstream Analysis for scRNAseq

Creation and visualization of different gene signatures (LSEC signatures) by upset plots was performed by the usage of R packaged UpSetR. Gene ontology analysis based on biological processes was analyzed using R package clusterProfiler for all gene signatures. The *AddmoduleScore* function in Seurat (version 4) was used to quantify gene signature scores of all LSEC signatures for each cell population. The gene signature score represents the average expression of all genes of the healthy or damaged LSEC gene signature within a cell population subtracted by the average expression of randomly selected genes within the same population.

### Whole Transcriptome Analysis

Paired-end sequencing on RNA of LSECs isolated using FACS from healthy and $CCl_4$ treated mice generated a fastq file for each sample. A quality control was performed before and after trimming using FastQC (www.bioinformatics.babraham.ac.uk/projects/fastqc) and AfterQC (34) followed by mapping all reads using STAR (35) to the mouse genome GRCm38.p6. Assembly was performed on every hit using StringTie and further analyzed by R package DESeq2 (36) for normalization and statistical analysis. Principle component analysis was performed using basic R functions and visualized by R package ggplot2. The expression of a selection of genes was validated using qPCR (**Supplementary Figure 2A**). qPCR was performed as previously described (27) and primers used for qPCR are displayed in **Supplementary Figure 2B**. For microarray data, CEL files were imported using R packages oligo (37) or affy (38) and normalized by Robust Multichip Average (RMA) algorithm.

### Gene Set Enrichment Analysis (GSEA)

GSEA Subramanian et al. (39) was performed on normalized counts using molecular signature databases Reactome, Biocarta and KEGG pathways. GSEA for bulk seq of LSECs was performed by comparing all groups (LSECs isolated from mouse livers after 1, 3, and 7 days $CCl_4$ injection) to LSECs from healthy mouse livers. All enriched pathways with a NES (normalized enrichment score) higher than 1 or lower than −1 with FDR lower than 0.25 were imported in Cytoscape and transformed into a network using EnrichmentMap (40). Pathways clustered together were named manually, based on overlapping functions, following the

protocol of Reimand et al. (41). Pathway clusters that change over time were manually summarized into a hypothetical graph created in Illustrator, based on the number of pathways within a cluster and changes after $CCl_4$ injection. GSEA using LSEC enriched gene sets was performed on normalized counts of healthy and liver diseases or on LSECs isolated from healthy or $CCl_4$ recovered livers. Following comparisons were performed to analyse LSEC signatures in advanced diseased livers vs. control groups: Hepatitis B (HBV) F3-4 vs. HBV F0-1 (GSE84044) (42), non-alcoholic steatohepatitis (NASH) F3–F4 vs. F0–F1 (GSE49541) (43), alcoholic steatohepatitis (ASH) vs. alcoholic steatosis liver (GSE103580) (44), advanced cirrhosis vs. healthy (GSE6764) (45), advanced hepatocellular carcinoma (HCC) vs. normal tissue (GSE6764) (45).

### Data Availability

Bulk RNAseq data of isolated LSECs after $CCl_4$ treatment has been deposited in the GEO public data base under accession number: GSE180366.

## Statistics

One-tailed Kruskal Wallis with Dunnett's multiple comparisons test was applied for the statics of ALT measurements, CD32b, Lyve1 and Lyve1/Ki67 stainings. Calculations were made using GraphPad Prism 9. Ns $> 0.05$, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

# RESULTS

## Enriched Genes in LSECs From Healthy and Cirrhotic Livers Identify LSECs in Advanced Cirrhotic Liver Diseases

To identify the presence of healthy or dysfunctional LSECs in RNA profiling data sets from human or mouse livers we set out to identify genes that are enriched in LSECs from healthy or cirrhotic livers. To this end, we used scRNAseq data of healthy and cirrhotic human livers reported by Ramachandran et al. (25). First, we identified LSECs and ECs expressing known LSEC and EC markers in healthy livers (**Figure 1A**; **Supplementary Figure 3**). Next, we identified an additional cell population which was not present in the endothelial cell population of healthy livers (**Figure 1B**). These cells were $CD34^{+}PLVAP^{+}VWA1^{+}$ positive which strongly resembled the scar-associated endothelial cell population identified by Ramachandran et al. (25). These cells were restricted to cirrhotic livers, expressed pro-fibrogenic genes and displayed an immunomodulatory phenotype (25). We refer to this population as damaged LSEC/ECs (**Figure 1B**) as some of the markers expressed in this population show a sinusoidal expression pattern in cirrhotic livers (25) but damaged ECs cannot be excluded. Subsequently, we defined genes that were higher expressed in healthy LSECs or damaged LSEC/EC population compared to all other liver cells (endothelial cells, macrophages, stellate cells, cholangiocytes, innate lymphoid cells (ILC), dendritic cells, T and B cells, hepatocytes and plasma cells) from healthy and cirrhotic livers with a fold change of at least two, and every gene should be expressed in at least 50% of cells within the healthy LSEC or damaged LSEC/EC population. This resulted in, respectively, 60 and 48 genes that were higher expressed in LSECs from healthy- or cirrhotic human livers in comparison to other liver cell types (**Figures 1A,B**). To identify genes that can further distinguish LSECs from healthy or diseased livers, we performed differential expression analysis between both populations resulting in a list of genes expressed higher in healthy LSECs compared to damaged LSEC/ECs from cirrhotic livers (**Figure 1C**). By combining genes that are enriched in LSECs from healthy or cirrhotic livers vs. other cells with genes that are higher expressed in one of the conditions vs. the other, we could create two genesets: a geneset for LSECs from healthy livers ($n = 48$) and a geneset for damaged LSEC/EC from cirrhotic livers ($n = 15$) (**Figure 2A**; **Supplementary Table 1**). Next, we performed gene ontology analyses to summarize the overlap in biological functions of genes included in each geneset. Genes that were enriched in healthy LSECs were part of GOs that are related to scavenging function and viral entry, both important characteristics of LSECs (46, 47). Genes that are enriched in damaged LSEC/EC belong to GOs that are related to dysfunctional LSECs, such as vascular development, migration and matrix organization, which are typical features of liver fibrosis (48, 49) (**Figure 2B**).

Next, we wondered whether we could use these gene sets to visualize an enrichment of damaged LSECs/ECs in microarray gene expression data from human livers. We therefore performed gene set enrichment analysis (39, 50) (GSEA) with the two LSEC gene sets on microarray gene expression data from human livers with different etiologies to identify the presence of healthy or damaged LSECs/ECs in advanced cirrhotic liver diseases. **Figure 2C** shows that gene sets that were highly expressed in healthy LSECs were substantially enriched in transcriptomes of healthy livers and diseases livers with early stage liver fibrosis (F0–F1). Gene sets that were highly expressed in damaged LSEC/ECs were enriched in cirrhotic livers (vs healthy livers) (45); ASH (vs alcoholic steatosis) (44), HCC (vs normal tissue) (45), NASH (F4–F3 vs. F0–F1) (43) and HBV (F3–4 vs. F0–1) (42) (**Figure 2D**). Taken together, our analysis suggests that LSECs transform into a more damaged endothelial cell phenotype in all advanced liver diseases that we investigated.

## Dynamic Response of LSECs to $CCl_4$-Induced Acute Liver Injury

LSECs play a crucial role in the regenerative response after an acute injury that can either lead to liver regenerative or a maladaptive fibrotic response (17). Yet, all studies and datasets we have used so far only reflected chronic liver injury. Therefore, we wanted to know whether the gene sets could also demonstrate LSEC phenotype changes after an acute injury. To this end, acute liver injury in mice was induced with a single dose of $CCl_4$ and livers were collected at 1, 3, and 7 days after injection (**Figure 3A**). Blood analysis shows acute liver injury (high ALT levels) at 24 h after a single dose of $CCl_4$, which decreases to baseline levels at day 7 (**Figure 3B**). Hematoxylin eosin staining shows necrotic areas that appear at 1 day and are more pronounced after 3 days demonstrating that liver injury is still present at that time point (**Figure 3C**). However, after 1 week the liver appears to have recovered from the injury. When we further examine

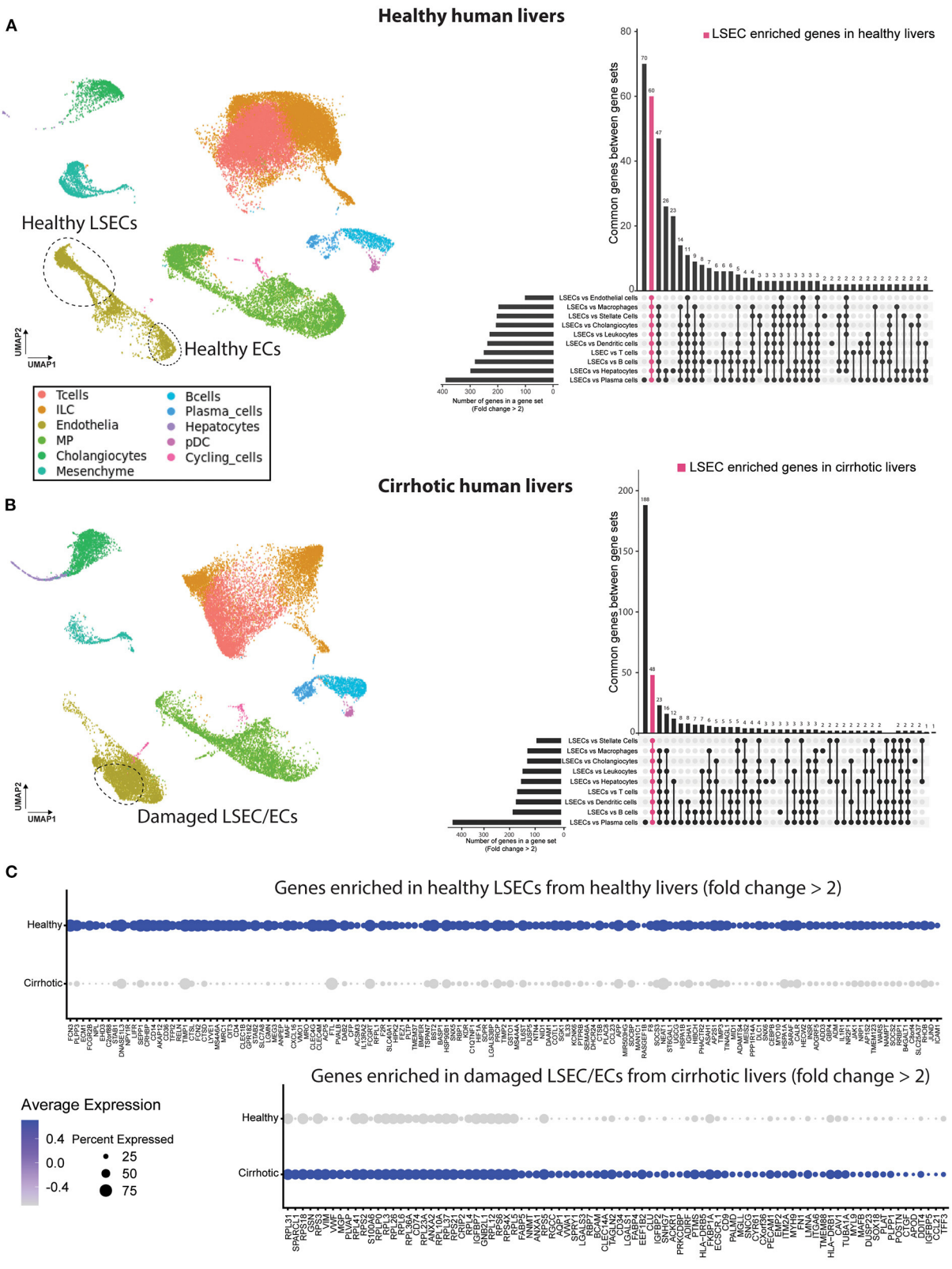


**FIGURE 1 |** Identification of genes higher expressed in LSECs in healthy and cirrhotic human livers. Left a UMAP plot of scRNAseq data of healthy **(A)** and cirrhotic **(B)** liver cells (25). Right an upset plot of differentially expressed genes (fold change > 2) in LSECs or damaged LSEC/ECs compared to all other cell types. Pink color represents LSEC enriched genes in healthy livers (60 genes) or damaged LSEC/EC enriched genes in cirrhotic livers (48 genes). **(C)** Dotplot of differentially expressed genes between LSECs of healthy and cirrhotic livers with fold change >2. All results were obtained with the use of the dataset of Ramachandran et al. (25).

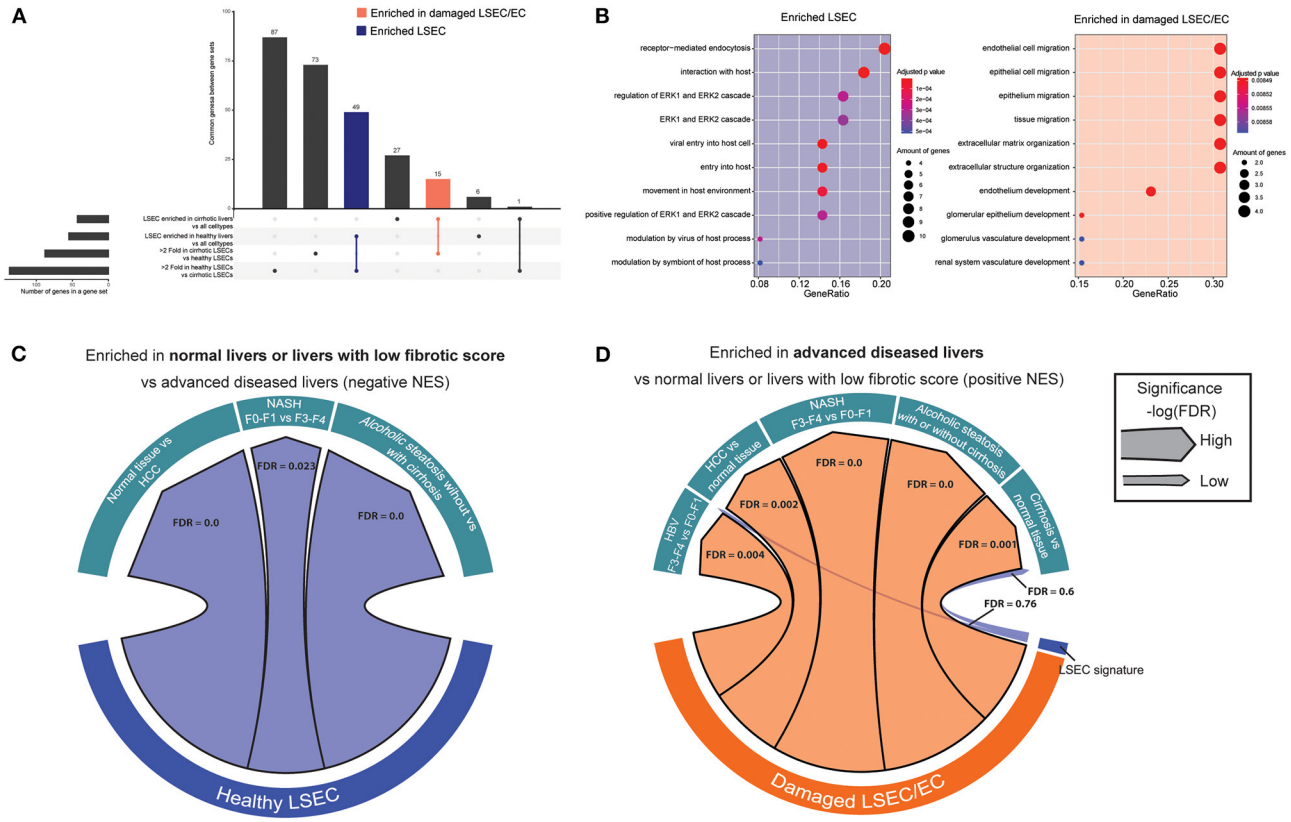


**FIGURE 2 |** Enriched gene sets in LSECs from healthy and cirrhotic livers identify healthy and damaged LSECs in chronic liver diseases. **(A)** Upset plot that combines genes that are differentially expressed between LSECs from healthy livers and damaged LSEC/ECs from cirrhotic livers and genes enriched in LSECs when compared to all other cell types in healthy and cirrhotic livers. Results were obtained with the use of the dataset of Ramachandran et al. (25). **(B)** Gene ontology analysis (biological processes) on enriched genes from LSECs and damaged LSECs/ECs. **(C,D)** Chord diagram of GSEA analysis [significance, –log(FDR)] of enriched genes from LSEC or LSEC/ECs in advanced liver diseases: cirrhotic livers (vs healthy livers) Wurmbach et al. (45), ASH (vs alcoholic steatosis) Trépo et al. (44), HCC (vs. normal tissue) Wurmbach et al. (45), NASH (F3–4 vs. F0–1) Murphy et al. (43) and HBV (F3–4 vs. F0–1) Wang et al. (42).

LSECs through staining, we see an increased trend of Lyve1 protein levels indicating that LSECs are still present and sinusoids are intact. However, we observed a temporary loss of CD32b expression after 1 and 3 days of $CCl_4$ treatment, indicating at least partial LSEC dysfunction which is restored after 1 week.

To further analyse LSECs after acute liver injury, livers were collected and LSECs were isolated via FACS at 1, 3, and 7 days after $CCl_4$ administration (**Figure 3A**; **Supplementary Figure 1B**) and transcriptome analysis was performed on the freshly isolated LSECs. Four samples were included for each condition apart from LSECs after 1 day of $CCl_4$-treatment, because 2 samples did not meet the quality standards for RNA sequencing (low RIN values). Although this reduces the statistical power, still more than 2,000 genes were differentially expressed when compared to healthy LSECs (**Supplementary Figure 4**). Principal component analysis (PCA) demonstrates separated clusters for each timepoint indicating a change in LSEC transcriptome after exposure to $CCl_4$ (**Figure 4A**). Interestingly, LSECs appear not to restore to the healthy LSEC cluster after $CCl_4$ induced injury, indicating that LSECs after 1 week $CCl_4$ have a different phenotype compared to healthy LSECs. Next, pathway analysis was performed on LSECs from $CCl_4$-treated livers compared to healthy LSECs (40). All enriched pathways were clustered in Cytoscape (**Supplementary Figure 5**) and graphically represented in **Figure 4B**. Shortly after the induction of acute liver injury, several pathways related to ROBO signaling and inflammation become significantly enriched (NES $>$ 1, FDR $<$ 0.25). After 3 days, pathways involved in angiogenesis, ECM (extra cellular matrix) production and cell cycle are induced. Interestingly, a considerable amount of cell cycle pathways are strongly active after 3 days of $CCl_4$ but seem to become inactive again after 7 days. This was confirmed by the presence of $Ki67^+Lyve1^+$ positive LSECs in livers 3 days after $CCl_4$, indicating that indeed LSECs are proliferating at day 3, but not anymore after 7 days (**Figure 4C**). Pathways regarding ECM production were elevated after 3 days of $CCl_4$ and remained elevated after 7 days. NCAM signaling, important for the inhibition of fibroblast growth factor signaling (51), shows a similar trend. One of the dysregulated ECM genes is Collagen 4 which has been described to be produced by LSECs (52–54). Upon acute injury we indeed see

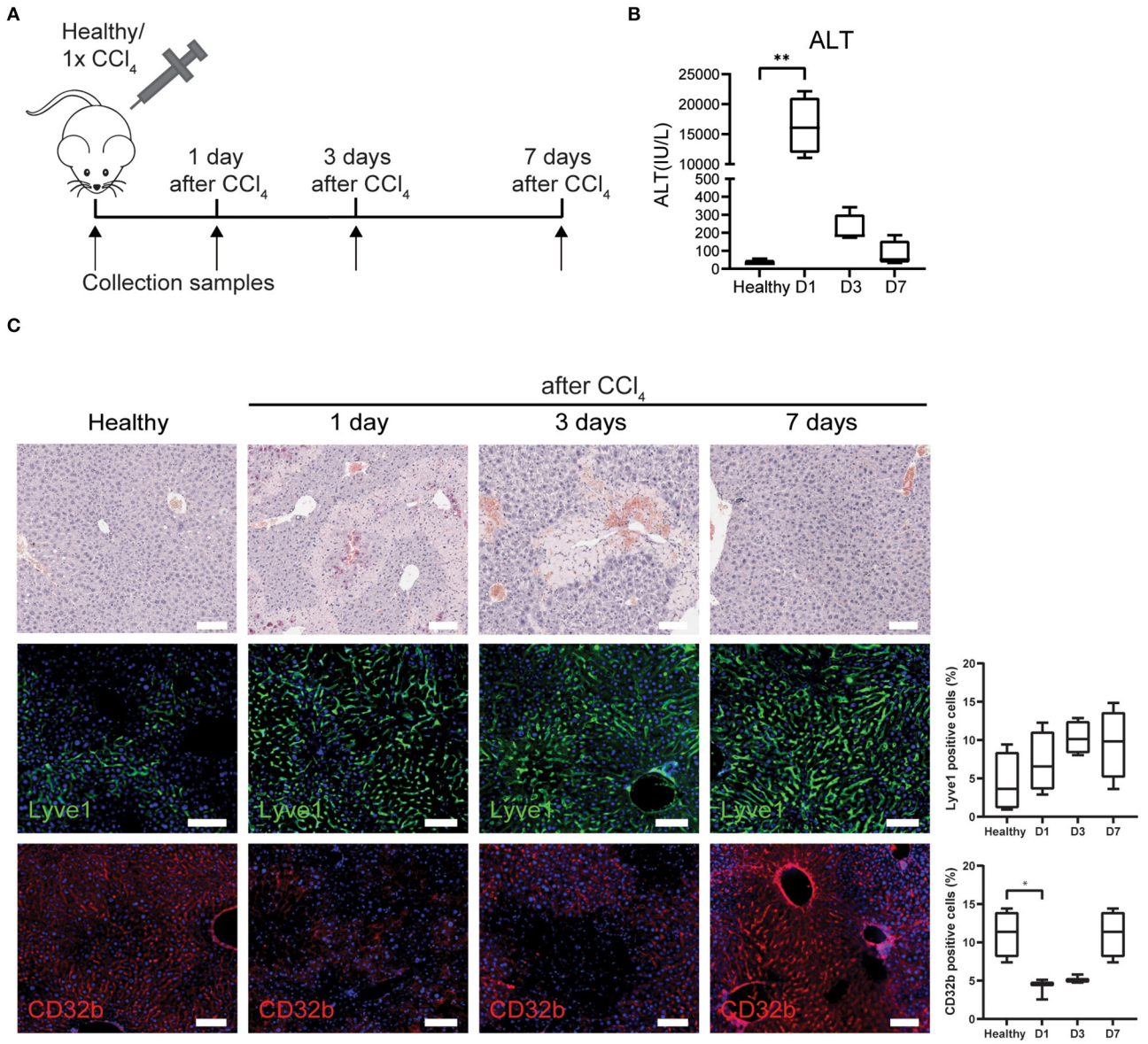


**FIGURE 3 |** LSECs in healthy livers or acute liver injury. **(A)** Isolation of LSECs from healthy livers and from livers after 1, 3, and 7 days after $CCl_4$ injection. **(B)** ALT levels of healthy mice and mice that received $CCl_4$ $^{**}P \leq 0.01$. **(C)** Immunohistochemistry with hematoxylin eosin staining and immunofluorescence staining and quantification of Lyve1 and CD32b ($^{*}P \leq 0.05$) on livers from healthy mice or mice that received $CCl_4$ (bar = 100 μm).

an induction of Collagen 4 expression on day 3, which shows a sinusoidal pattern (**Figure 4C**).

## Generation and Validation of an LSEC- and a Damaged LSEC Signature

To compare human with mouse LSEC dysfunction, we analyzed the expression of human LSEC gene sets (**Figures 1**, **2**) in mouse LSECs after an acute $CCl_4$-induced liver injury. Genes that are enriched in healthy human LSECs show diverse expression patterns in mouse LSECs after acute liver injury (**Figure 5A**). Typical LSEC genes such as *STAB2* and *CLEC4G* are downregulated upon liver injury, in contrast to genes such as *LYVE1*, *CLEC1B*, and *CD36* which are upregulated at early timepoints, indicating that these genes cannot always discriminate healthy LSECs from damaged LSECs. Genes that were expressed higher in healthy LSECs were selected for the generation of a restricted healthy LSEC signature that should identify healthy LSECs in mice and human samples. This signature contains both novel (*PLPP3*, *NTN4* and *OIT3*) and well-established (*CLEC4G* and *STAB2*) genes for LSECs which show a high expression in healthy human LSECs (**Figure 5B**). To generate also a more restricted gene signature that can specifically identify LSECs in damaged livers instead of both damaged LSECs and ECs, we first identified genes that were differentially

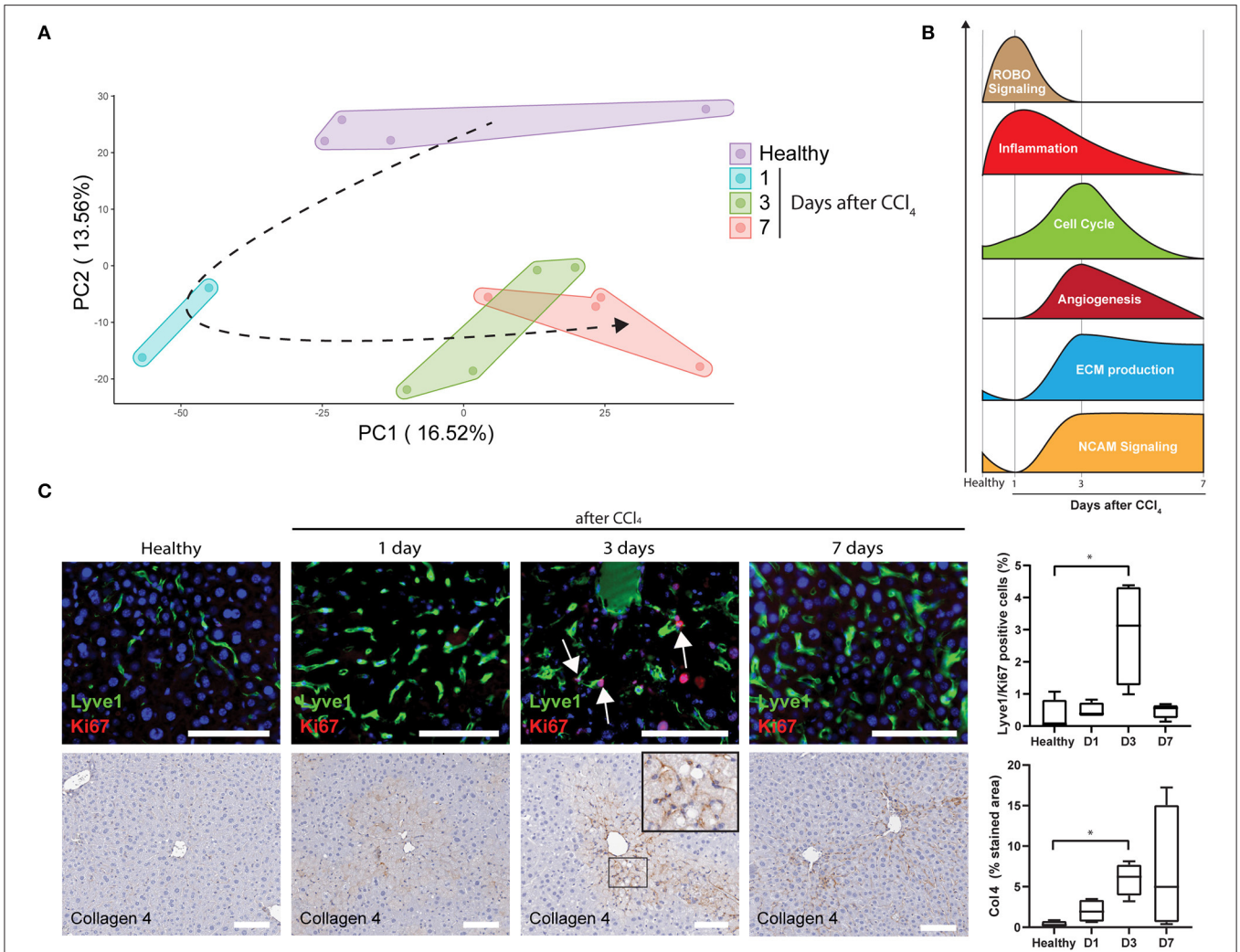


**FIGURE 4 |** Upregulated pathways in LSECs during acute liver injury **(A)** PCA of LSECs from healthy livers and livers after an acute injury by $CCl_4$ administration. **(B)** Schematic representation of pathway analysis from LSECs isolated after $CCl_4$ administration. **(C)** Immunofluorescence staining of Lyve1/Ki67, immunohistochemistry staining and quantification of Collagen 4 (*$P \leq 0.05$) and Lyve1/Ki67 positive cells (*$P \leq 0.05$) on healthy livers and livers after $CCl_4$ administration (bar = 100 μm).

expressed in the damaged LSEC population in comparison to healthy endothelial cells (**Figure 5C**). These differentially expressed genes were compared to previously identified enriched gene sets from damaged LSECs/ECs (**Figure 2A**) which resulted in a damaged LSEC signature that contained only four genes: *Fabp4/5* and *Vwf/a1* (**Figure 5D**). These four genes were all upregulated in $CCl_4$-induced liver injury after 1 day or 3 days. Moreover, these four genes are highly expressed in the damaged human LSEC population of the Ramachandran et al. (25) data set (**Figure 5E**). The expression of two healthy and damaged LSEC signature genes were validated using qPCR and confirmed the RNAseq data (**Supplementary Figure 2A**).

Next, we wanted to examine if scRNAseq data sets of LSECs from healthy and diseased livers can be identified as such with these two LSEC signatures. As samples can differ quite a lot between studies due to a different definition of healthy subjects, different isolation methods, different scRNAseq approaches, different etiologies and species we validated our newly generated LSECs signatures in 4 independent scRNAseq data sets of healthy human livers (21, 24) and healthy or diseased (NASH and fibrotic) mouse livers (26, 32) (**Supplementary Figure 6**). Using the LSEC signature we could show a higher gene signature score in LSEC-related populations in healthy human livers compared to all other cell populations (**Figures 6A,B**). Moreover, the damaged LSEC signature shows a low gene signature score in all liver cell types in healthy human livers except for a slightly higher gene signature score for periportal LSECs and (portal) ECs. These results confirm that quantification of LSEC signatures (scores) can be used to identify LSECs in scRNAseq data of human healthy livers. Unfortunately, we could not validate our signatures in a different scRNAseq data set of cirrhotic patients due to the lack of publicly available human data. Next, we validated the LSEC signatures using scRNAseq data of healthy and diseased (NASH and fibrotic) mouse livers

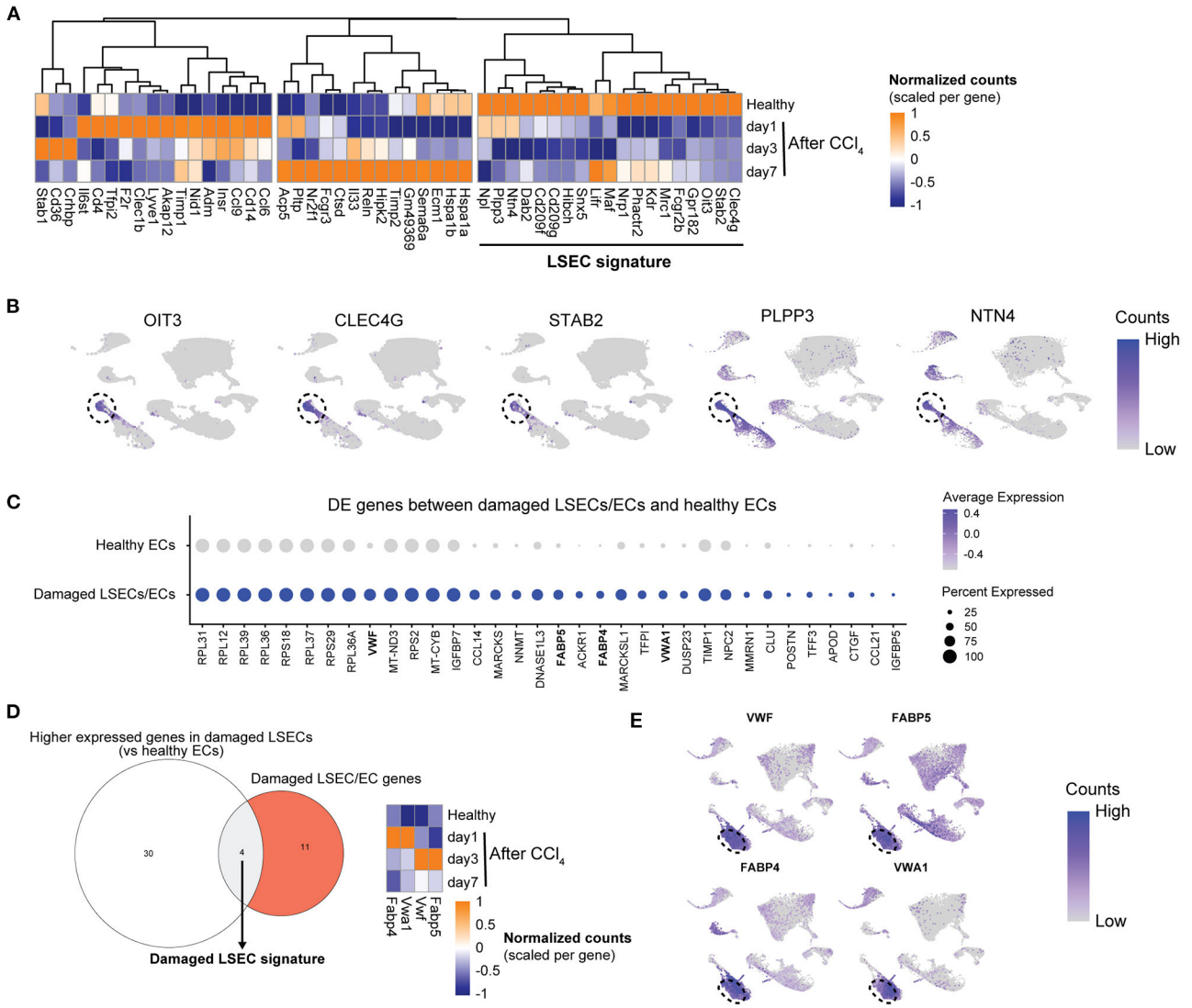


**FIGURE 5 |** Development of an LSEC and a damaged LSEC gene signature. **(A)** Heatmap of the enriched healthy human LSEC genes in LSECs from healthy and acutely injured mouse livers. The genes used for the LSEC signature are underlined. **(B)** UMAP plot of Ramachandran et al. (25) dataset with gene expressions in healthy livers for LSEC signature genes with the LSEC population marked by the dotted line. **(C)** Dot plot of differentially expressed genes between damaged LSEC/EC population and healthy ECs from Ramachandran et al. (25). **(D)** Venn diagram of genes higher expressed in damaged LSECs from **(C)** and damaged LSEC/EC signature. The damaged LSEC signature is represented by the overlapping region. Heatmap of LSEC signature genes in LSEC from healthy and acutely injured livers. **(E)** UMAP plot of Ramachandran et al. (25) dataset with gene expression from damaged LSEC signature genes in cirrhotic livers. Damaged LSEC population is marked by the dotted line.

(26, 32). In both data sets, the LSEC/EC populations from control livers have a high LSEC signature score, but is also still present (but lower) in NASH and fibrotic livers. More importantly, the damaged LSEC signature score is higher in LSEC/EC population from NASH livers, and to a lesser extend in $CCl_4$ livers, indicating that LSECs are damaged and can be identified in NASH and fibrotic livers using these 4 genes (**Figures 6C,D**). These findings demonstrate that the LSEC signatures can be used to identify and distinguish damaged LSECs from healthy LSECs in scRNAseq data of human and mouse livers.

## DISCUSSION

LSECs are important for liver homeostasis and play a pivotal role in both acute and chronic liver injury by influencing HSCs and other cell types in the liver. ScRNAseq studies identified numerous EC populations and revealed well-established and novel LSEC markers for LSECs in healthy and disease states. However, most studies use one specific mouse model (12, 26, 32) or only human cirrhotic livers (25). In this study we sought to generate LSEC signatures that can identify healthy and damaged LSEC populations in multiple transcriptome data sets. We first

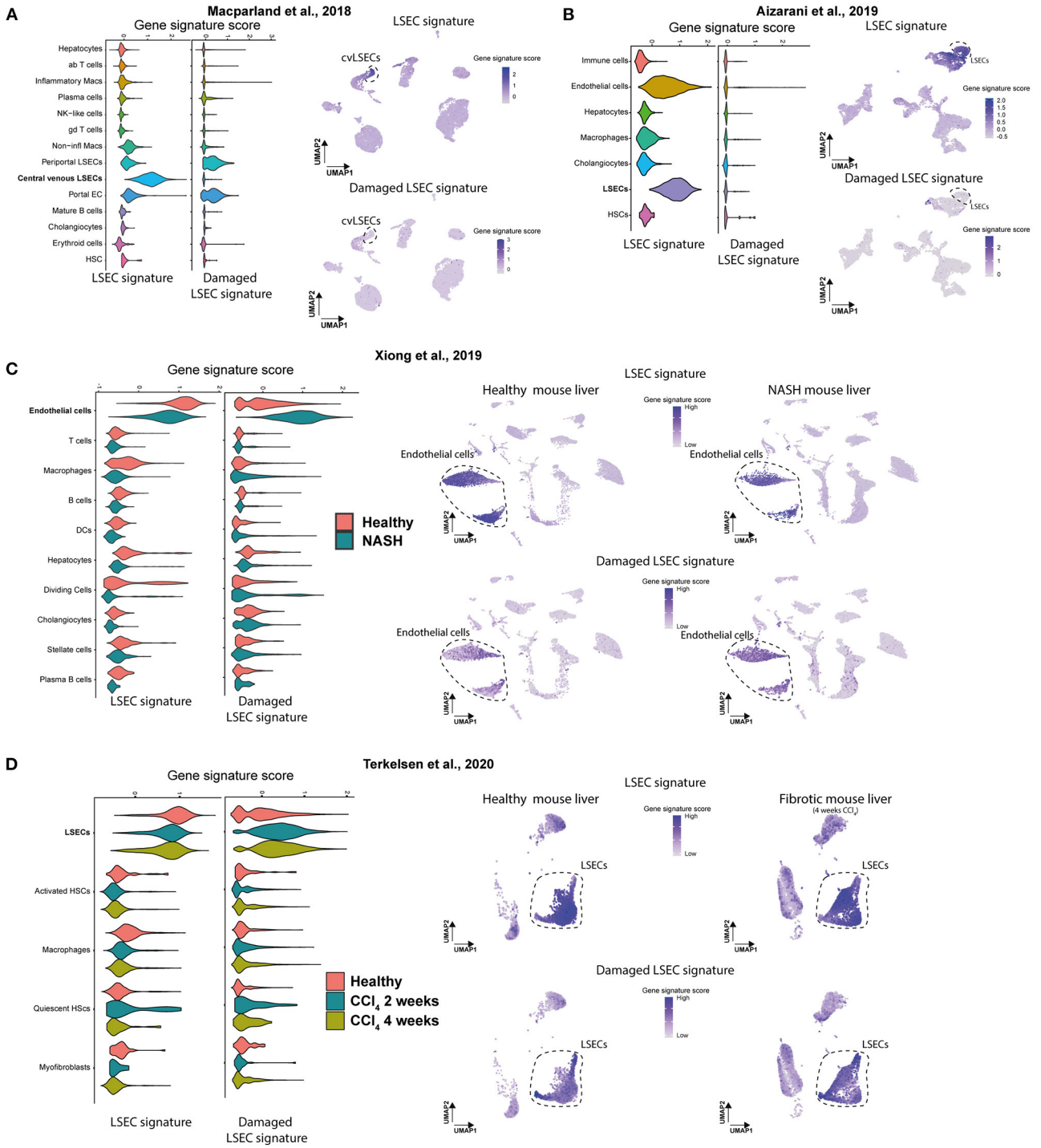


**FIGURE 6 |** Gene signature scores of LSEC signatures in scRNAseq data of human and mouse livers. **(A,B)** Gene set enrichment score (Violin plot left) of both signatures and UMAP plot of scRNAseq data of healthy human livers with gene set enrichment score of both signatures in purple (right). Results were obtained with the use of the dataset of Macparland et al. (24) and Aizarani et al. (21). **(C,D)** Gene set enrichment score (Violin plot, left) of both signatures and UMAP plot of scRNAseq data of (NASH and fibrotic) mouse livers with gene set enrichment score of both signatures in purple (right). Results were obtained with the use of the dataset of Xiong et al. (26) and Terkelsen et al. (32).

focussed on genes enriched in LSEC or damaged LSEC/EC in the human liver scRNAseq data of Ramachandran et al. (25). Using these enriched human gene sets we could show that in cirrhotic livers of patients suffering from HBV, HCC, ASH, and NASH there is a clear enrichment of damaged LSECs. Subsequently, we showed that during acute liver injury in mice certain LSEC specific genes are quickly downregulated which resulted in a more specific LSEC signature that can identify healthy LSECs in mouse and human scRNAseq data. Finally, we developed a damaged LSEC signature comprised of Fabp4/5 and Vwf/a1 that can identify damaged LSECs in transcriptome data of NASH and fibrotic mouse livers.

In this study we used $CCl_4$ to induce an acute liver injury and to evaluate whether the transcriptional changes that occur in LSECs in chronic liver disease already occur upon acute liver damage. After an acute liver injury we observed that LSECs quickly change their phenotype by upregulating Lyve1, by temporarily downregulating CD32b, proliferating and upregulating ECM genes after 3 days of $CCl_4$. Previous studies showed that LSECs can produce a basement membrane during chronic liver injury by deposition of Collagen 4 and Laminin (52, 53, 55). Interestingly, in the data of Ramachandran et al. (25), we also see the expression of *COL4A1* and *COL4A2* mainly in the damaged LSEC/EC population (**Supplementary Figure 7**) indicating that primarily LSECs express *COL4A1* and *COL4A2* in chronically injured human livers. Here, we could show that Collagen 4 deposition is already initiated during acute liver injury.

Here we defined an LSEC signature that contains several well-established LSEC markers such as the scavenger receptors *STAB2*, *CLEC4G*, *CD209*, *MRC1*, and *CD32B* (*Fcgr2b*) but also receptors important for VEGF signaling such as *KDR* and *NRP1* (**Figure 5**). The expression of some of these markers (*STAB2* and *CLEC4G*) has been shown to decrease during chronic liver disease (47). Other genes in this signature are less known but have been mentioned mainly in gene profiling studies (*OIT3*, *NPL*) (24, 56, 57). Genes that showed a higher expression after acute liver injury in mice were not included in the LSEC signature, such as *LYVE1* and *STAB1*. However, we would like to note that these genes could still be useful markers because the induction is scaled per gene, meaning that there is an induction of expression but this induction could be insignificant if the expression of that certain gene is already very high in the LSEC population. There were several other LSEC enriched genes, such as *CLEC1B*, *CD14*, *IL33*, and *CCL6/9*, that showed an induction after acute liver injury and that have been mentioned in other gene profiling studies. This indicates that inflammation could play a role in LSECs during acute liver injury. *TIMP1* and *TIMP2*, often associated with HSCs, also show an induction. Further analysis of these genes in data from Ramachandran et al. (25) showed a strong expression of *TIMP1* and *TIMP2* in LSECs and endothelial cells from human livers indeed showing that these cells do express *TIMP1* and *TIMP2* (**Supplementary Figure 8**). However, TIMP1 and 2 were not expressed in LSECs or endothelial cells from scRNAseq data from Xiong et al. (26). The damaged LSEC signature contains the known capillarization marker *VWF*, and genes *VWA1*, *FABP4*, and *FAPB5*. Further investigation of the literature shows that protein expression of these signature genes are indeed associated with a damaged LSEC phenotype in mice and human. For example, *FAPB4*, also known as (adipocyte) fatty acid binding protein 4, was recently found to be upregulated in LSECs during liver fibrosis, can promote LSEC capillarization and is suggested to be a key regulator involved in the onset and progression of fibrosis in two liver fibrosis models in mice (58). In addition, FABP4 is also overexpressed in patients with HCC (59). Multiple studies have shown that vWF is not expressed by LSECs in healthy livers but is increased in LSECs during fibrosis in several animal models, for example after $CCl_4$ treatment in mice and rats (60, 61), and NASH with or without cirrhosis in rats (62). Moreover, $vWf^{+}$ LSECs were significantly correlated to the fibrosis stage in patients with cirrhosis (63) and a higher vWF expression has been linked to old age and pseudocapilarization (64). Targeting LSECs to alleviate fibrosis through one of these 4 genes could be an option as it was recently shown that the treatment with the *FABP4* selective inhibitor BMS309403 alleviated lipopolysaccharide induce acute liver injury and high fat diet-induced NASH in mice (65), and a knockout of *FABP4* reduces fibrosis in $CCl_4$ and bile duct ligation model in mice (58).

The use of microarray or bulk-seq profiling data can mask the fact that the gene expression signal detected represents only a small portion of a total LSEC population. Few dedifferentiated or damaged LSECs could be responsible for the enrichment of the damaged LSEC/EC gene sets. To obtain more insight into the abundance of dysfunctional LSECs in human and (damaged) mouse livers, more specific LSEC gene signatures were validated in scRNAseq datasets. In this study scRNAseq datasets of different liver disease models were used; two healthy human scRNAseq data sets (21, 24) and two mouse healthy and NASH/fibrotic data sets (26, 32). The recent dataset from Su et al. (12) was not included due to a potential contamination of duplets, making incorporation of this dataset in this study problematic (data not shown). In healthy human livers, the LSEC signature separates LSECs from other liver cells, and only a low signature score is present for periportal LSECs and portal ECs when the damaged LSEC signature is used. Nevertheless, it remains difficult to separate portal and central endothelial cells from portal and central LSECs as they cluster strongly together because LSECs still express endothelial markers such as *CD31* or *CD105* even though these markers have been reported to be lower in LSECs (11, 12). In both healthy and NASH/fibrotic mouse livers, the LSEC signature was abundantly expressed even though the gene signature score is clearly lower in NASH/fibrotic livers which suggests that LSECs partly lose their phenotype in chronic liver disease. More importantly, the damaged LSEC signature had clearly a high gene signature score in all cells of the LSEC/EC population of NASH livers which indicates that all LSECs are damaged in NASH/fibrotic mouse livers. Further scRNAseq analysis of acutely injured mice or human livers would shed more light on the independent changes of different endothelial and LSEC populations and could give more insight into early mechanisms of LSEC-dysfunction or capillarization. A next step in this research could be a larger prospective sequencing effort on biopsy material of livers at different stages of chronic liver disease, or recovering from liver disease, to evaluate whether

one can correlate the rise of a healthy LSEC signature to the improvement of liver fibrosis while a certain level of the damaged LSEC signature can predict progression of the liver disease. Some proteins from the damaged signature could be measured in blood and correlated to the development of fibrosis. For example FABP4 in the blood is already positively correlated to the fibrosis stage and inflammatory grade in patients with NAFLD and NASH (66). In addition, protein levels of the damaged LSEC signature genes could serve as biomarkers for the extent of LSEC damage in acute liver injury, as LSEC damage occurs in ischemia-reperfusion, drug-induced liver injury and hepatic sinusoidal obstruction syndrome (67). For instance, one study showed that FABP4 was elevated in the serum of mice with acute liver injury induced by a single injection of LPS (65). Moreover, in patients with acute liver injury and acute liver failure, vWF is elevated in the serum, but could not be correlated to poor disease outcome (68). One should note that in this study vWF levels could have been affected as blood samples were also collected after NAC administration.

To conclude, we showed that the transcriptome of LSECs transform into a cirrhotic transcriptome independent of the etiology in multiple microarray datasets from human livers. In addition, two unique LSEC signatures were developed and validated in several independent scRNAseq datasets, demonstrating that these signatures can recognize LSECs in healthy and chronically injured livers. Moreover, using several scRNAseq data sets we showed that all LSECs isolated from NASH/fibrotic mouse livers have a damaged LSEC expression profile. These results indicate that during mouse and human chronic liver disease, the change of LSECs toward a cirrhotic dysfunctional phenotype is strong and highlights the potential of LSECs as a therapeutic target for chronic liver disease.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

## ETHICS STATEMENT

The animal study was reviewed and approved by Ethical Committee of Animal Experimentation of the Vrije Universiteit Brussel.

## AUTHOR CONTRIBUTIONS

SV and EO: conceptualization, investigation, methodology, formal analysis, validation, visualization, data curation, and writing—original draft. VD: investigation and formal analysis. NE: methodology and formal analysis. IM: investigation and methodology. LG: conceptualization, funding acquisition, data curation, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.750044/full#supplementary-material

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# The Contribution of Liver Sinusoidal Endothelial Cells to Clearance of Therapeutic Antibody

*Bethany H. James[1], Pantelitsa Papakyriacou[1], Matthew J. Gardener[2], Louise Gliddon[2], Christopher J. Weston[1] and Patricia F. Lalor[1]**

[1] Centre for Liver and Gastroenterology Research and National Institute for Health Research (NIHR) Birmingham Biomedical Research Centre, Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom,
[2] Antibody Pharmacology, Biopharm Discovery, Glaxo Smith Kline Research and Development, Stevenage, United Kingdom

Many chronic inflammatory diseases are treated by administration of "biological" therapies in terms of fully human and humanized monoclonal antibodies or Fc fusion proteins. These tools have widespread efficacy and are favored because they generally exhibit high specificity for target with a low toxicity. However, the design of clinically applicable humanized antibodies is complicated by the need to circumvent normal antibody clearance mechanisms to maintain therapeutic dosing, whilst avoiding development of off target antibody dependent cellular toxicity. Classically, professional phagocytic immune cells are responsible for scavenging and clearance of antibody *via* interactions with the Fc portion. Immune cells such as macrophages, monocytes, and neutrophils express Fc receptor subsets, such as the FcγR that can then clear immune complexes. Another, the neonatal Fc receptor (FcRn) is key to clearance of IgG *in vivo* and serum half-life of antibody is explicitly linked to function of this receptor. The liver is a site of significant expression of FcRn and indeed several hepatic cell populations including Kupffer cells and liver sinusoidal endothelial cells (LSEC), play key roles in antibody clearance. This combined with the fact that the liver is a highly perfused organ with a relatively permissive microcirculation means that hepatic binding of antibody has a significant effect on pharmacokinetics of clearance. Liver disease can alter systemic distribution or pharmacokinetics of antibody-based therapies and impact on clinical effectiveness, however, few studies document the changes in key membrane receptors involved in antibody clearance across the spectrum of liver disease. Similarly, the individual contribution of LSEC scavenger receptors to antibody clearance in a healthy or chronically diseased organ is not well characterized. This is an important omission since pharmacokinetic studies of antibody distribution are often based on studies in healthy individuals and thus may not reflect the picture in an aging or chronically diseased population. Therefore, in this review we consider the expression and function of key antibody-binding receptors on LSEC, and the features of therapeutic antibodies which may accentuate clearance by the liver. We then discuss the implications of this for the design and utility of monoclonal antibody-based therapies.

**Keywords: liver, endothelium, antibody, therapy, disease**

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*Natalia A. Osna, University of Nebraska Medical Center, United States*

***Reviewed by:***
*Srivatsan Kidambi, University of Nebraska-Lincoln, United States*
*Manuel Romero-Gómez, Seville University, Spain*

****Correspondence:***
*Patricia F. Lalor*
*p.f.lalor@bham.ac.uk*

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# INTRODUCTION

## The Growing Importance of Therapeutic Antibodies

Monoclonal antibody-based therapies for a variety of conditions have been available since the late 1980s. Therapeutic antibodies are biopharmaceuticals that recognize and bind to a specific antigen leading to either activation or inhibition of downstream biological pathways. Monoclonal antibodies (mAbs) are the most common clinical tool and represent the leading treatment modality for diseases ranging from inflammatory and autoimmune disease to cancer. Upon recognition of cognate antigen they either trigger an antibody mediated cellular cytotoxic (ADCC) and/or a complement-dependent cytotoxic (CDC) effector response, or act to neutralize the intended target antigen. Antibodies are large molecules, which generally don't interact with transport molecules or detoxification enzymes, exhibit ion channel-related complications or cause immunogenicity. Thus antibody-based therapeutics tend to be potent and well tolerated (Catapano and Papadopoulos, 2013). Only three antibodies were approved by the FDA in 2013 and four in 2014, whereas as of December 2019 a total of 79 mAbs have met approval standards with over 500 currently undergoing clinical trials around the world (Kaplon et al., 2020). Hence the global therapeutic antibody market is predicted to generate over $300 billion by 2025 (Lu et al., 2020).

However, adverse effects post-treatment are not uncommon, and often relate to the pathway being targeted or the mode of action of the drug itself. Importantly problems and adverse events are not always predicted by preclinical screening strategies. Toxicity or adverse events may relate to biological function of the target molecule [e.g., minor bleeds in patients treated with anti-platelet agents such as abciximab (Tamhane and Gurm, 2008)] or interaction with off-target tissues. Less specific toxicity can also be explained by hypersensitivity responses to immunogenic "non" human elements of therapeutics. When designing a new antibody-based therapy there is also a need to minimize interactions with non-target molecules and tissues other than the therapeutic target. These issues can be resolved by careful engineering of antibody to reduce immunogenicity, maximize efficacy, and minimize clearance. Similarly, choice of administration route has an impact on its efficacy and clearance. Intravenous administration rapidly delivers 100% of antibody into the systemic circulation and generates high plasma concentrations, but increases the potential for off target exposure, hypersensitivity reactions and the cost of in-house treatment. In contrast, sub-cutaneous and intra-muscular administration deliver antibody *via* the lymphatic system. Here formulation, injection volume and physical factors such as age and weight of the patient (Richter et al., 2012; Richter and Jacobsen, 2014) can impact on bioavailability. Antibodies destined for use in chronic conditions need to have the longest possible half-life and minimal clearance rates to support a favorable administration strategy and ensure dosing frequency is not prohibitive. Importantly preclinical pharmacokinetic testing of new reagents in a disease specific model is vital to ensure patient demographics for likely clinical use are best represented. In this article we will consider the underestimated role of the liver, and specifically the sinusoidal endothelial cells in antibody clearance. We also consider strategies that could be utilized to minimize hepatic clearance, and the impact of age or chronic disease on endothelial: antibody interactions. We begin with a review of therapeutic antibody generation and structure before considering implications for hepatic targeting and explanations for reported adverse events in clinical use.

## Generation of Antibodies for Therapeutic Use

Therapeutic mAbs have similar structure to endogenous immunoglobulin, i.e., four polypeptide chains, two light and two heavy, each with both a Fab fragment and an Fc region. These form a complex Y-shaped structure (see **Figure 1**). The Fab fragment is composed of one constant region and one variable domain which make up the antigen binding site. The Fc region at the tail end of the antibody binds to elements of the immune system such as complement components and surface receptors known as Fc receptors (FcRs). Historically, man-made antibodies were generated using the hybridoma technique (Kohler et al., 1976) to generate murine monoclonal reagents as exemplified by OKT3 (Kung et al., 1979). This murine antibody targeting human CD3 antigen on T cells was widely used in immunotherapeutic contexts including management of allograft rejection. However, it has since been withdrawn due to side effects and generation of host anti-murine antibodies which reduced efficacy (Sgro, 1995). Subsequently, the disadvantages of murine mAbs were partially overcome by generation of chimeric antibodies. Here recombinant DNA technology was used to generate hybridized reagents consisting of the variable region from a mouse antibody fused to a human antibody constant region. This reduced the potential for the generation of anti-murine antibodies. The first chimeric mAb approved by the FDA, abciximab (Lu et al., 2020) is a Fab fragment antagonist to glycoprotein IIb/IIIa receptor used to inhibit platelet aggregation. This was soon followed by, the first full length IgG chimeric antibody "rituximab," an anti-CD20 antibody widely used as an immune modifier (Maloney et al., 1997). To further reduce the risk of immunogenicity, the residual proportion of mouse antibody has been further diminished by the advent of complementarity determining region (CDR) grafting approaches (Riechmann et al., 1988; Tsurushita et al., 2005). Despite the increased proportion of human sequence within such antibodies, adverse reactions still occurred (Nechansky, 2010).

This led to a drive to produce fully humanized reagents through application of technologies such as phage or yeast display of antibody peptide libraries (Smith, 1985; McCafferty et al., 1990). This method is rapid and robust with libraries containing $1 \times 10^{10}$ antibody fragments available and is now considered the gold standard for recombinant antibody production. The anti-TNF antibody Adalimumab was generated using this approach and is currently one of the best-selling therapeutics in the world, generating $20 billion in 2018 (Kempeni, 1999; Lu et al., 2020). Similarly, immunization of transgenic rodents to generate fully humanized antibodies is significant. Here the mouse IgG

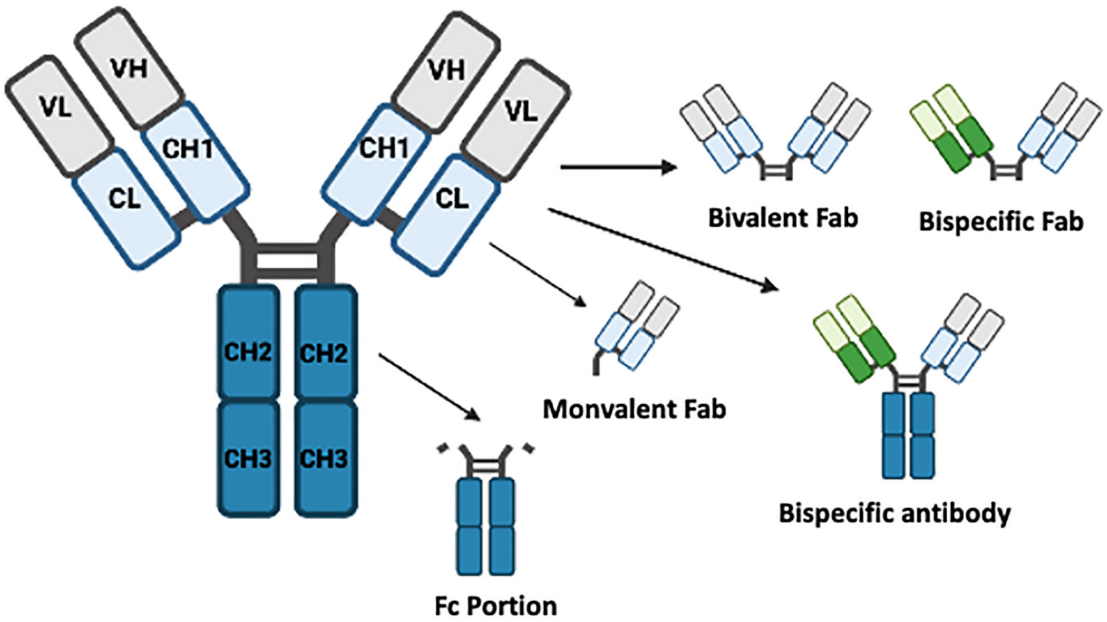


**FIGURE 1 |** Typical structure of monoclonal and bispecific antibodies. Monoclonal antibodies (left structure) are composed of four polypeptide chains, two light (L) and two heavy (H), each both a Fab fragment and an Fc region (blue) joined by a hinge section to create a Y-shaped structure. The Fab fragment which recognizes antigen is composed of constant (C) and variable (V) domains which make up the antigen binding site. Specific fragments are also shown. Fab fragments can be bivalent or monovalent, and engineered bispecific antibodies can contain or lack an Fc portion.

gene repertoire is replaced with human counterparts leading to development of transgenic lines (Lonberg et al., 1994; Mendez et al., 1997) such as the Xeno-mouse. The huge potential of this technology is exemplified by panitumumab, the first Xeno-mouse reagent to gain FDA approval. This fully human $IgG_2$ EGFR antibody is used in therapy for metastatic colorectal cancer (Jakobovits et al., 2007). Currently 19 approved mAbs have been developed using such transgenic mice. This method is advantageous as there is often no requirement for an affinity maturation step for targets with high affinity, and full-length IgG antibodies are made. However, if the antigen being used to immunize is particularly toxic then phage display is the preferred technique. To date human and humanized mAbs are the dominant format of therapeutic antibodies accounting for, respectively, 51 and 35% of all mAbs currently in clinical use (Lu et al., 2020).

Whilst traditional monoclonal antibodies bind to a single antigen, bispecific tools have been engineered to improve targeting [increase the efficacy of immune: target cell or receptor:ligand interactions (Kang and Lee, 2021)] and exhibit favorable tissue penetration. Different formats exist and each has its own advantages and challenges. Fragment based bispecific antibodies (BsAb) lack a Fc region but still contain two independent antigen binding domains. As there is no Fc region present, these BsAb are considerably smaller than traditional mAbs allowing them to penetrate tissues easily. A good example of this approach is blinatumomab used in treatment of lymphoblastic leukemia (Kantarjian et al., 2017). This antibody combines two antigen receptor epitopes to recognize CD3+ effector T cells and CD19+ B cells to stimulate recognition and elimination of B cell blasts. Although effective at improving survival, this approach is not without adverse events including elevation in liver enzymes (Kantarjian et al., 2017). The other formulation is the full-length IgG-like asymmetric BsAb (Fc-based BsAbs, or BsMabs) which retain an Fc portion. Mosunetuzumab used in treatment of leukemia exemplifies this approach again targeting both a B cell epitope (CD20) and CD3 (Schuster, 2021), and also bears a Fc domain engineered to minimize FcγR and complement binding. However, if a strong immune response is required, intact Fc regions facilitate interactions with FcR and C1q. The small size and dual antigen specificity of such bispecific reagents places a target cell in close proximity to the effector cells resulting in a more effective response than more traditional mAbs. Hence such forms of BsAb have low therapeutic concentrations and short half-life, (Wang et al., 2019) which can meant that frequent infusions are required possibly increasing potential for off target effects. More recently there have been attempts to improve specificity of targeting by using gene therapy approaches to drive cell specific expression of bispecific antibodies at the site of need. This is particularly attractive if hepatospecific targeting is required, given the high phagocytic activities and ready absorbance of liposomes

and nanosomes within the liver. This approach is elegantly exemplified by the work of Kruse et al. (2017) who generated hepatitis B Ag : CD3 specific bispecific antibodies with antiviral efficacy *in vivo* (Kruse et al., 2017).

## A Focus on Fc Receptors and Mechanisms of Antibody Uptake and Clearance

Highly charged cationic molecules like antibodies with poor pharmacokinetic profiles are cleared reasonably quickly (Haraya et al., 2019) and evidence suggests that this clearance takes place in highly vascularized organs like the liver and spleen (Li et al., 2014). The liver in particular is a major site for internalization and catabolic clearance of therapeutic antibodies as they are typically too large for renal elimination. This is facilitated in part by an impressive scavenging system. Cells of the hepatic reticuloendothelial system express many receptors that can bind and internalize antibodies either by target mediated clearance or *via* non-specific uptake. As noted above, Fc receptors on a cell surface generally recognize the Fc portion of antibody and as a consequence activate and modulate immune responses or clear immune complexes. This could take the form of destruction of an opsonized target cell or the activation/regulation of cellular effector responses. However, exaggerated antibody-dependent autoimmune and hypersensitivity responses and circulating therapeutic antibody pharmacokinetics are also impacted by the action of these receptors (Hogarth and Pietersz, 2012). In the context of antibody-based therapies, interaction with FcR is important for specific targeting of an immune response. The Fc gamma receptor (FcγR) family of proteins consists of six FcγRs in humans which include FcγR1 (CD64), FcγRIIa,b and c (CD32a-c) and FcγRIIIa and b (CD16a and b) (Brooks et al., 1989). Each has a slightly different cellular distribution and affinity for IgG (Hogarth and Pietersz, 2012). Human IgG1 and 3 bind more effectively to FcγRs than IgG2 and 4 (Schwab et al., 2015) but IgG1 antibodies are still the most commonly used for therapies (Lucas et al., 2018). Clustering of antibody and target antigen may be enhanced by binding to FcγRIIb (Stopforth et al., 2016). In contrast, internalization, and catabolism of antibodies *via* FcγR may be particularly important for antibodies with circulating soluble antigens or which form large immune complexes with target as these tend to bind well to FcRs (Lucas et al., 2018).

Engagement of receptor on immune cells generally induces a cellular response *via* activation of immunoreceptor tyrosine-based activation motif (ITAM) and SRC family kinase activation. In most cases this causes a pro-inflammatory response, but FcγRIIb has inhibitory effects *via* activation of immunoreceptor tyrosine-based activation motif (ITIM) (Hogarth and Pietersz, 2012), despite binding IgG with a relatively low affinity. In B cells this can downregulate signals from the other FcR and cause apoptosis. There are also descriptions of two variants of FcγRIIb (b1 and b2) which have slight differences in the ability to internalize antibody due to variance in structure of the cytoplasmic domain of the receptor (Stopforth et al., 2016). The neonatal Fc receptor (FcRn) seems to be more involved in antigen presentation and IgG recycling within cells. It is expressed by endothelium (Vaccaro et al., 2005), tissue macrophages and Kupffer cells, enterocytes and some epithelial cells (Latvala et al., 2017). It is atypical in that along with binding IgG it also recognizes albumin and plays key roles in transcytosis and recycling of both to maintain circulating concentrations (Pyzik et al., 2019).

The process for uptake and recycling of antibody is described in **Figure 2**. Once bound to FcγR a monoclonal antibody is internalized into an endosome. Here they encounter membrane bound FcRn (Roopenian and Akilesh, 2007) which is responsible for the protection of IgG catabolism, recycling the antibody to the surface leading to an increased half-life. This binding is pH dependent and will only occur in acidic endosomes, with a pH at around 6–6.5. FcRn containing vesicles become exposed to an increasing pH gradient until they reach the cell surface and physiological pH. This causes the mAb and FcRn to dissociate and the antibody is then released from the cell and recycled back into circulation. mAbs that fail to be recycled by FcRn are either cleared *via* the activation of C1q, and undergo clearance *via* the classical complement pathway or are degraded by proteases present within lysosomes (Leipold and Prabhu, 2019). Therefore the FcRn is important to spare the mAb from degradation and prolong the half-life (Haraya et al., 2019) potentially reducing therapeutic dosing and frequency. Some studies have suggested that it is FcRn that primarily impacts on pharmacokinetics and that FcγRIIb has little impact on circulating antibody distribution (Abuqayyas et al., 2013). However, it is important to note that some studies with knockout animals deficient in FcγRIIb tested antibodies at concentrations far below therapeutic concentrations. Even in these circumstances there was an increase in liver distribution (albeit variable) even at low dose suggesting that within the liver FcγRIIb may be involved in clearance and degradation of antibody (Abuqayyas et al., 2013). This seems to be particularly important for antibody: antigen complexes which are cleared into liver whilst antigen alone is not (Ljunghusen et al., 1990). Thus, in the next section we describe the function of the hepatic sinusoidal endothelial cells to highlight their potential roles in antibody bioavailability.

## Liver Sinusoidal Endothelial Cell Structure and Function

One factor which remains challenging in the development of antibody therapies relates to their pharmacokinetics and clearance in tissue. This alters exposure to target antigen and ultimately efficacy. Distribution within a tissue is impacted upon by movement across the vessel wall and interaction with endothelial cells and macrophages which express the receptors described above. Tissues like the liver which have fenestrated non-continuous endothelial cells, are highly perfused and abundantly vascularized, will have greater exposure to antibody (Datta-Mannan, 2019). The isoelectric point of an antibody appears to particularly influence hepatic clearance, such that engineering of antibody variants with high pI leads to preferential sequestration and clearance by the liver (Ganesan et al., 2012). Transport of antibody from blood into tissue is dependent on local perfusion gradient and key features of the

vessel wall such as presence of fenestrated endothelium and basal lamina thickness. Junctional structure is also important with the presence of endothelial cells containing tight junctions limiting access, as is seen in the brain (Tabrizi et al., 2010). Thus, the liver sinusoidal bed presents a particular challenge. Liver sinusoidal endothelial cells (LSEC, **Figure 3**) which are exposed to both systemic and portal blood are designed to maximize the exchange of useful material from the blood into the liver and vice versa (Shetty et al., 2018). They form part of the hepatic reticuloendothelial system with roles in both the clearance of detrimental pathogens and waste products and the transport of important metabolic products to and from the proximal hepatocytes. These activities are facilitated by the presence of numerous macroscopic pores or "fenestrations," organized into sieve plates which transverse the full thickness of the endothelial layer allowing transport of lipids and proteins (Hunt et al., 2019) and also medicinal drugs such as lidocaine and paracetamol (Mitchell et al., 2011). Importantly unlike the kidney (Satchell and Braet, 2009) and other organs, the hepatic sinusoidal endothelial fenestrations lack a diaphragm and basal lamina. This, plus the ability of cells to rapidly regulate fenestration diameter and number (O'Reilly et al., 2010; Cogger et al., 2016) further regulates transport.

Liver sinusoidal endothelial cells also express an unusual complement of scavenger receptors which recognize, bind, and rapidly internalize an enormous diversity of extracellular ligands (Shetty et al., 2018). These are characterized into classes A to J depending on their ligand recognition and structural properties (Patten et al., 2021) and LSEC express receptors in classes SR-B, E, F, G, and H to support clearance of fatty acids, lipids ECM proteins, glycosaminoglycan molecules and apoptotic cells. This significant endocytic capability supports the immune regulation (Knolle and Limmer, 2001), metabolic capacity (Li et al., 2011) and "waste management" (Smedsrod, 2004) functions of the liver. In the context of this article, it is important to note that LSEC also express high levels of FcR under homeostatic conditions. The FcR on LSEC can bind opsonized pathogens and macromolecules to facilitate clearance, with blood-borne immune complexes rapidly cleared from the circulation by both Kupffer cells (KC) and LSEC (Smedsrod, 2004). Although KC may be more efficient at clearing immune complexes, the increased number of LSEC compared to KC within a liver means that their total capacity may be similar (Johansson et al., 2000). Circulating immune complex clearance can cause tissue damage and inflammation in some conditions (Johansson et al., 2000) and thus sinusoidal endothelial cells contribute to the process of clearance *via* the FcR interaction (Johansson et al., 2000). This may be particularly important when the load of circulating IgG is high (Johansson et al., 2000). LSEC have been suggested to express all three of the major Fcγ receptors (Smedsrod, 2004) and it is estimated that up to 75% of all the FcγRIIb within the body is expressed on LSEC (Ganesan et al., 2012). Thus, this abundant receptor expression plays a key role in removal of small immune complexes from blood. We have documented expression in human livers (**Figure 4**) and confirm that expression is abundant and localized to LSEC in the healthy liver. Expression is maintained in chronic disease (**Figure 4**) but the distribution is altered in cirrhosis and intensity of staining is reduced, which may suggest an impact on function. FcRn has a more widespread hepatic distribution, described to be present on epithelial cells, endothelium, and immune cell populations (Pyzik et al., 2019) in animal studies. In agreement, our investigation of human liver (**Figure 5**) confirms intense sinusoidal expression localized to Kupffer cells. Periportal immune cells are also positive with a degree of intracellular staining in hepatocytes. Faint intracellular LSEC staining is confirmed by confocal studies (**Figure 5** final panel) on cultured human LSEC. Although historically the role of FcRn LSEC has not been well documented (Skogh et al., 1985), hepatocyte intracellular FcRn (Pyzik et al., 2019) has been linked to clearance and catabolism of antibody and albumin transport. Interestingly we also see intracellular localization in human hepatocytes (**Figure 5**) with increased peri-membranous distribution in advanced disease (Blue arrows **Figure 5**). This may reflect a response to hypergammaglobulinemia in cirrhosis and liver disease (Alonso et al., 2012; Cacciola et al., 2018). FcRn also plays roles in the pathology of toxic liver injury. Drugs including paracetamol are transported bound to circulating albumin, and blockade of the interaction between albumin and FcRn reduces hepatotoxicity after paracetamol administration (Pyzik et al., 2017). Interestingly LSEC also express a scavenger receptor lectin, dendritic cell specific ICAM-3 grabbing non-integrin (DC-SIGN) (Lai et al., 2006; Schwab and Nimmerjahn, 2013) which has been demonstrated to be a coreceptor for some viruses (Gramberg et al., 2007). This receptor also bind intravenously administered therapeutic Immunoglobulin (IVIg) (Hogarth and Pietersz, 2012; Schwab and Nimmerjahn, 2013), upregulates expression of FcγRIIb and protects against immune-complex mediated disease (Anthony et al., 2011).

All the evidence above suggests that in a healthy liver, the LSEC are armed with key receptors and endocytic machinery to bind and transport antibody and immune complexes. There is functional evidence to support this. For example, studies of clearance of Bispecific antibodies in cynomolgus monkeys suggest a role for both macrophages and LSEC in clearance (Datta-Mannan et al., 2016). Here use of clodronate to deplete macrophages did not have a great effect on antibody clearance, suggesting that the contribution of macrophages was marginal. This was confirmed by costaining of therapeutic antibody with markers of LSEC to confirm co-localization (Datta-Mannan et al., 2016) with little staining for the bispecific antibodies observed in macrophages. Studies of humanized mice which express human FcγR and are given a humanized antiplatelet antibody confirm these findings with no major effect after macrophage deletion (Schwab et al., 2015). Clearance of opsonized pathogen too is linked to intact FcγRIIb function on LSEC, with deficient mice exhibiting slower pathogen clearance (Ganesan et al., 2012). A more interesting question, however, is what impact LSEC have on the pharmacokinetics and pharmacodynamics of therapeutic antibodies? Also, whether newer antibody formulations can be optimized to exhibit the most favorable dosing profiles and minimize side effects by consideration of LSEC function in health and disease?

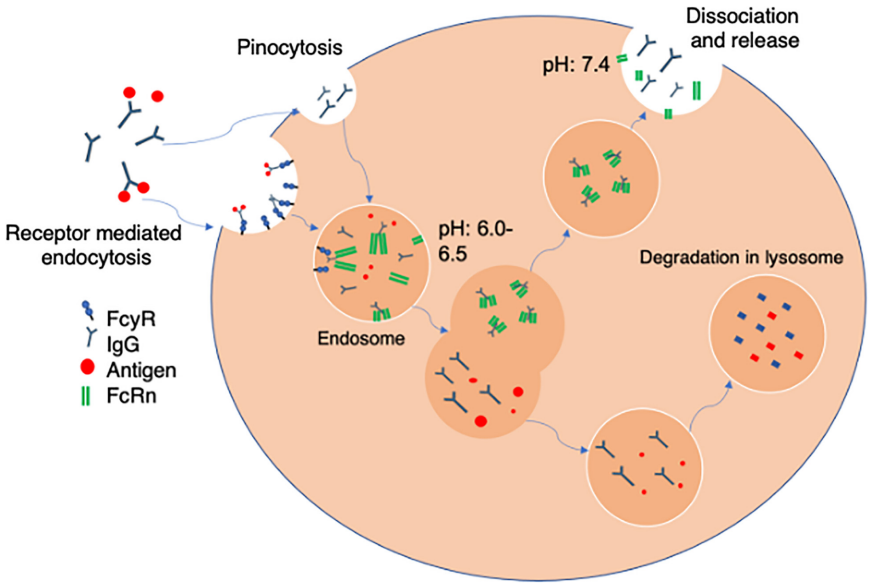


**FIGURE 2 |** Receptor mediated antibody uptake. The Fc portion of free antibody or antibody bound to soluble antigen to form an immune complex bind to FcγR at the cell surface. Once bound antibody is internalized into an acidified endosome *via* fluid phase pinocytosis. The endosomes contain FcRn which binds *via* the heavy chains in the Fc region in a pH sensitive manner. The FcRn can then recycle bound antibody back to the cell membrane where physiological pH of blood allows uncoupling and release back into the circulation. Alternately mAbs that fail to be recycled by FcRn are either cleared *via* the activation of C1q, and the classical complement pathway or are degraded by proteases present within lysosomes within the cell.

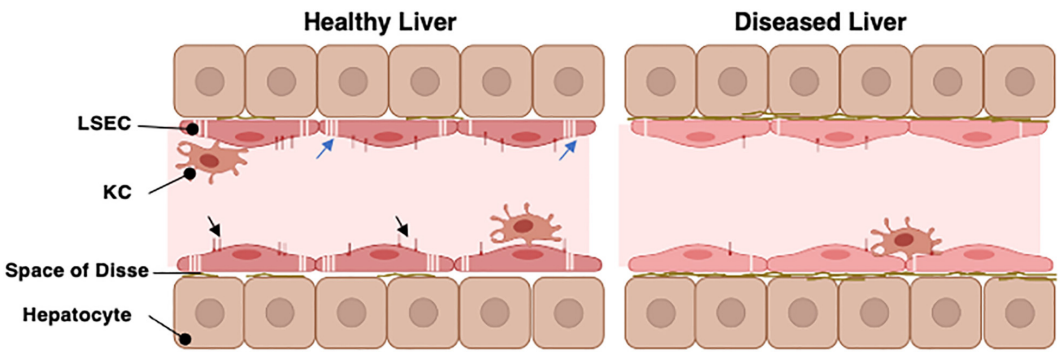


**FIGURE 3 |** The organization of the hepatic sinusoid. The hepatic sinusoids represent the capillary bed of the liver and are lined by specialized liver sinusoidal endothelial cells (LSEC). These sit above the hepatocyte layer separated only by the Space of Disse which contains minimal basement membrane in a healthy liver. LSEC have specialized pores in their cell surface (the fenestrations, blue arrows) which organize into sieve plates to facilitate direct exchange of materials between the hepatic parenchyma and bloodstream. The LSEC also express unique profiles of cell surface scavenger receptors and Fc receptors (Black arrowheads) which can interact with macromolecules within the slow flowing sinusoidal blood. Kupffer cells (KC) are specialized macrophages which patrol along the sinusoids to fulfil their immune regulatory functions. In chronic disease or aged livers, the nature of the LSEC changes. They lose most of their fenestrations and alter abundance of scavenger and Fc receptors. They also produce a more complex basement membrane. This restricts movement of materials into and out of the parenchyma.

## Does Liver Sinusoidal Endothelial Cells Biology Influence the Outcome of Therapeutic Antibody Administration, and Is This Important When Designing Antibodies?

Evidence cited above from knockout animals which have modified hepatic FcR expression confirm the contribution of the liver to clearance. Therapeutic antibody development approaches may include engineering of the Fc portion of humanized antibodies to enhance interactions with FcRn and improve pharmacokinetics. Fc receptor mediated clearance of immune complex is often a desirable therapeutic strategy. Here cell surface Fc receptors bind to the Fc portion of IgG antibodies in immune complexes with their target, and these are cleared from the circulation through uptake into macrophages and endothelial cells in the liver (Lovdal et al., 2000; Ganesan et al., 2012). However, in some situations internalization of therapeutic antibodies *via* actions of FcγRIIb can reduce clinical efficacy, as has been reported for the use of rituximab in some leukemias

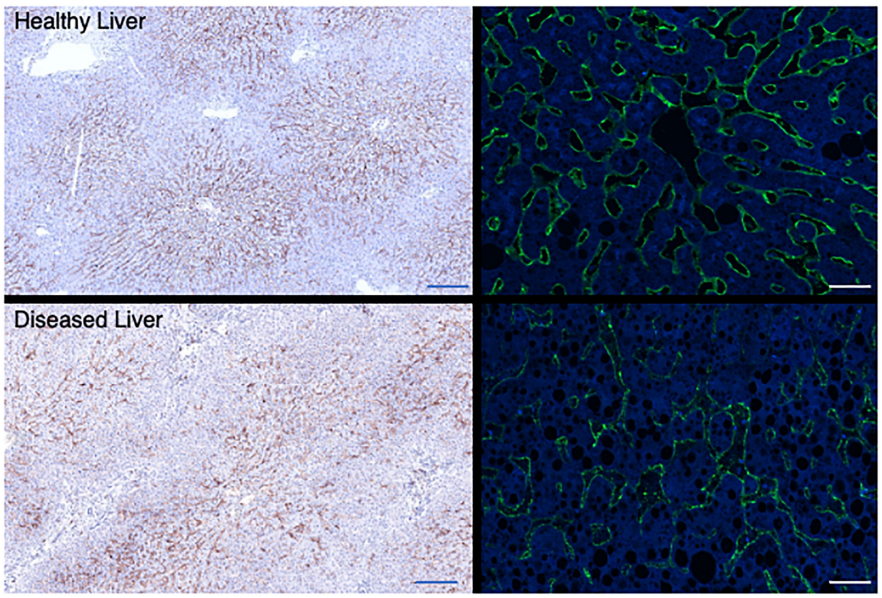


**FIGURE 4 |** Hepatic sinusoidal endothelial expression of FcγR2b alters in disease. Representative immunochemical (left panels, 10× original magnification Bar is 200 um) and immunofluorescent stains (right panels, 100× original magnification, Bar is 20 um) for FcγR2b on representative examples of healthy (top row) and diseased liver [bottom row, cirrhotic explanted liver from patient with PSC (left) or ALD (right)]. FcγR is localized to the LSEC in both cases, but expression is more intense and consistent across the sinusoid in a healthy context. In explanted cirrhotic human livers some areas of sinusoids lack expression completely.

(Lim et al., 2011) and cancer models (Clynes et al., 2000). It is also noteworthy that circulating immune complex clearance can cause tissue damage and inflammation in some conditions (Johansson et al., 2000). This may be particularly important when the load of circulating IgG is high (Johansson et al., 2000). For example, studies of Humanized DR-5 antibodies (an apoptosis inducing TNFR) with an engineered Fc fragment to enhance FcγRIIb binding in mice engineered to express human FcγRIIb, resulted in increased ALT/AST and mortality (Li and Ravetch, 2012) at supraphysiological doses. Here the FcR was important for the hepatotoxicity. In other studies, humanized antibody designed to target tumor cells by binding to a TNFR stimulatory receptor (CD137) on immune cells to promote anti-tumor immunity responses (Qi et al., 2019) such as Urlumab (Segal et al., 2017) was also associated with liver toxicity, inflammation and liver related adverse events. Mechanistic studies on such antibodies suggest that LSEC expression of FcγRIIb increases crosslinking and activatory effects of strong agonistic antibodies to enhance liver toxicity (Qi et al., 2019). However, engineering of Fab fragments that retain strong agonism minimizes this effect. It is also important to consider potential target-related toxicities alongside FcR-related hepatotoxicity in some cases. As an example, antibodies against TNF were tested as potential anti-inflammatory therapies in human alcoholic hepatitis but some studies were terminated due to adverse outcomes (Blendis and Dotan, 2004) or showed no mortality benefit over standard therapies. There are reports of drug induced toxicity associated with many formulations of anti-TNF antibodies (Lopetuso et al., 2018), particularly in patients with autoimmune liver disease (Tobon et al., 2007) and thus vasculotoxicity associated with antibody clearance could explain an underlying mechanism of damage. However, it is also important to note that TNFα plays a key role in hepatocyte regeneration (Fausto, 2000) and promotes hepatic infiltration by immune cells which drive repair (Chauhan et al., 2020) or fight sepsis which is a significant risk in alcoholic hepatitis (Sharma et al., 2009). Thus biological inhibition of hepatic repair mechanisms may also explain some of the adverse outcomes associated with this approach.

GSK305002 is a humanized IgG antibody that neutralizes the soluble chemokine CCL20 and was in development as a potential therapy for inflammatory disease (Laffan et al., 2020). Although no safety signatures appeared in a phase 1 study in humans, subsequent longer term escalating dose toxicity studies in cynomolgus monkeys highlighted a significant vascular inflammation in most subjects which is unexpected for an antibody targeting soluble antigen. In the liver this presented as moderate inflammation with immune deposits localized within the sinusoids. Target antigen did not appear to be contained in these deposits and importantly anti-human antibodies were not detected or were present at a level too low to explain the findings (Laffan et al., 2020). This would suggest that localization of FcR [or CCL20 (Shields et al., 1999)] on the LSEC may have provided a focus for immune complex deposition and complement mediated toxicity toward the LSEC. Vasculotoxicity has also been seen with other antibody drugs and can present as Sinusoidal Obstruction Syndrome (Jain and Litzow, 2018). This is damage to the sinusoidal endothelium, particularly in central areas of the lobule which exposes the subendothelial cells to blood constituents driving a necrotic response and vascular occlusion. This may relate to drug conjugates bound to antibodies

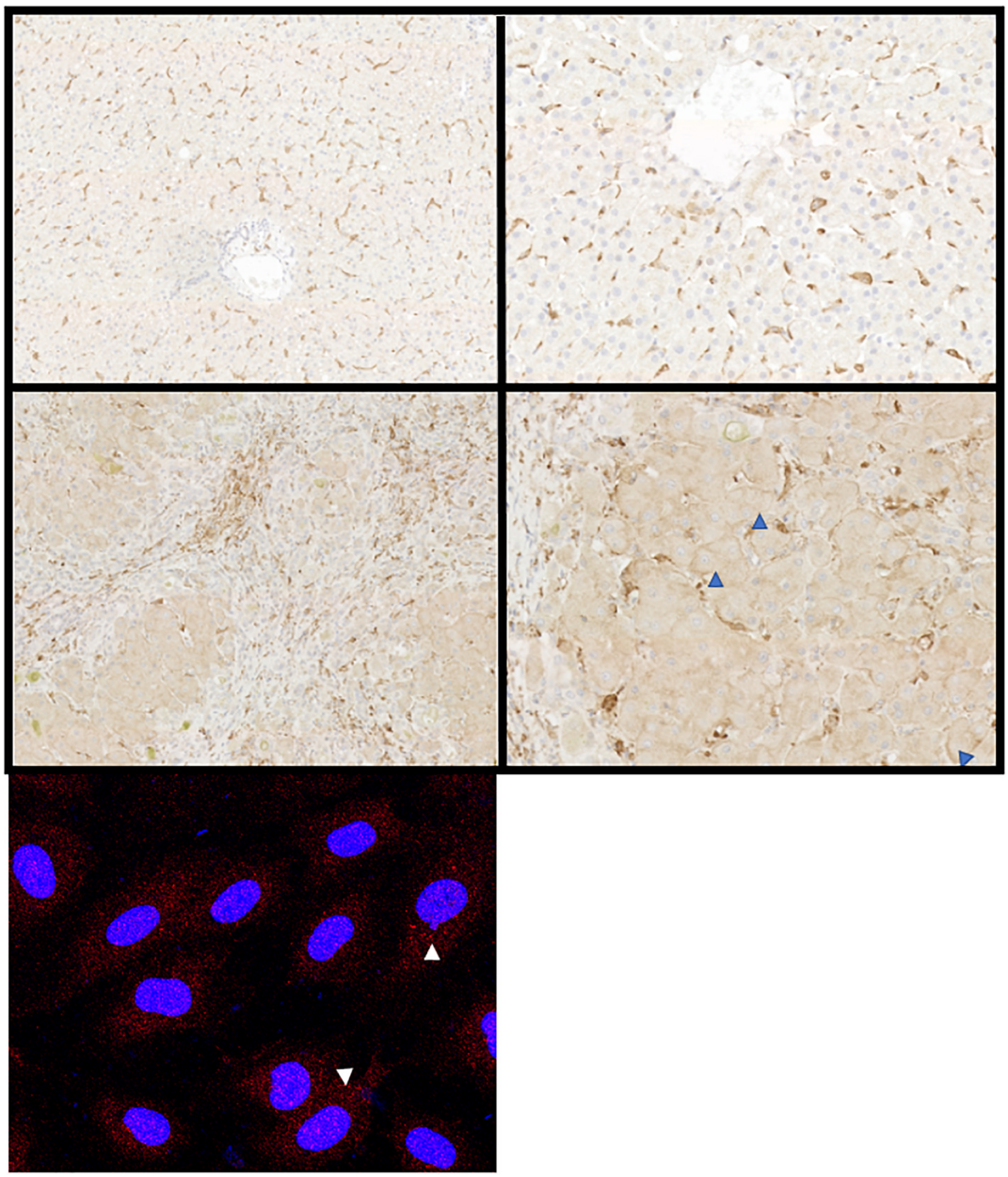

**FIGURE 5 |** Hepatic expression of FcRn alters in disease. Representative immunochemical (top panels), and immunofluorescent stains (bottom left panel) for FcRn on representative examples of healthy (top row) and diseased liver (middle row) or primary cultures of human LSEC. Both hepatocytes and sinusoidal cells express FcRn but the intensity increases in disease (ALD, middle row). Hepatocellular membrane expression increases as disease progresses (blue arrowheads). Original immunochemical stain images captured at 10× and 50× magnification (left and right panels, respectively). Cultured LSEC express FcRn (red stain) in an intracellular vesicular pattern (white arrows).

to facilitate target cell toxicity (e.g., calicheamicin for inotuzumab and gemtuzumab). Perhaps the best example of a serious adverse reaction to antibody therapy, the first human trials of the CD28 specific TGN1412 (Suntharalingam et al., 2006) also highlights how important FcR binding is and how hard responses are to predict. TGN1412 is a potent agonistic antibody developed for use in treatment of some cancers and rheumatoid arthritis. Its agonistic events are potentiated by interactions with Fc$\gamma$RIIb, particularly that expressed in B cells (Dudek et al., 2019), but presence of endothelial cells is necessary to recreate the immune activatory responses in *in vitro* assays (Dhir et al., 2012).

Immune or toxic responses to biotherapeutics are complex and can be target related or influenced by the structure and clearance of the antibody itself. For this reason, all new therapeutics are tested extensively in preclinical models and healthy volunteers before proof of efficacy in a patient. However,

there are still instances where preclinical models have failed to accurately predict human responses or those in a specific patient cohort or requirements for alternate dosing regimens in chronic disease. Hepatic impairment and impact on antibody kinetics may alter exposure, tolerability and effectiveness if metabolism or excretion is altered (Sun et al., 2020). This may relate to lower albumin production by a damaged liver impacting on antibody exposure of factors which alter expression or function of FcRn and FcγRs could also alter systemic exposure. However, regulatory bodies in some cases suggest that validation of MAb therapy in populations with renal or hepatic impairment is not vital for licensing (Lucas et al., 2018). Moreover, there are clear examples where prior liver injury or older age increase the risk of adverse events of antibody-based treatments (Jain and Litzow, 2018). This has meant that for some antibody-based therapies where hepatotoxic side effects have been noted, pre-existing clinical liver disease is considered an exclusion for use. For example - tocilizumab (humanized IL-6 receptor antibody) and anakinra (IL-1R antagonist antibody) used as anti-inflammatories in rheumatoid arthritis have potential, well described hepatotoxic consequences in some patients (Mahamid et al., 2011) particularly if other immunosuppressive drugs such as methotrexate have been administered.

**TABLE 1 |** Clinical challenges associated with hepatic clearance of biological therapies and strategies to mitigate risk during drug development.

| Clinical challenge | Explanation | Mitigating strategy |
|---|---|---|
| Impact of LSEC Fc receptors on antibody PK | Accelerated or delayed clearance of circulating antibody | Modify Fc portion to enhance interaction with FcRn and improve half life Modify Fc portion to minimize interaction with FcγRIIb |
| Localized hepatotoxicity or DILI in reponse to antibody therapy in humans | Enhanced deposition and clearance by LSEC leading to vasculotoxicity | Analysis of Fc portion and specific testing of clearance by human FcR to minimize crosslinking and activation in sinusoid |
| Complement mediated toxicity/Sinusoidal obstruction syndrome associated with antibody therapy | Immune complex binding to LSEC and cell apoptosis leading to exposure of basal lamina | Careful screening for binding to Fc receptors on LSEC |
| Altered antibody PK in older patients or patients with underlying liver disease | LSEC capillarization, reduction in hepatic albumin production | Careful screening for pre-existing disease in patient populations. Age-dependent pharmacokinetic assessment at Phase 1 testing |
| Complications due to autoantibody production in hepatic autoimmunity | LSEC capillarization or autoantibody occupancy of FcRs impacting on PK | Use of FcRn blockers to enhance IgG degradation |
| Desire to improve half life of therapeutic antibody | Accelerated clearance by hepatic FcγRIIb | Engineering of Fc portion to minimize interaction or delay internalization of receptor |
| Lack of clinical efficacy upon testing in human subjects | Reduced abilities of rodent or primate models to recreate human hepatic antibody clearance | Inclusion of human cell based or tissue array screens in pre-trail development stages |

The challenge remains being able to predict and explain such toxicities, and then to be able to engineer a solution to them. It is important to note that the FcγRs are slightly different in mice (Schwab et al., 2015) than humans and thus variations in human receptors not represented in mice can mean that rodent models are not perfect for predicting humanized antibody activity and clearance. Similarly, IgG4 mAbs don't interact with monkey FcR's and thus wouldn't be picked up in species specific screens (Hansel et al., 2010). Even in a human context, individuals have polymorphisms in Fc: FcR interactions which underpin interindividual variation in antibody clearance and efficacy (Hansel et al., 2010). Levels of FcR expression change with age and disease state. We note above that FcRn expression within the liver is altered in cirrhosis and suggested this could relate to circulating antibody concentration fluctuations in disease (Holdstock et al., 1982) which is clearly associated with poor prognosis (Cacciola et al., 2018). However, it may also be a consequence of age or disease related sinusoidal capillarization (**Figure 3**). Importantly not all scavenger receptors on LSEC decrease with aging or capillarization. Thus whilst receptors such as CD36 are increased on LSEC with age or development of fatty liver disease (Sheedfar et al., 2014), expression of mannose receptor decreases (Dini et al., 1990) and studies in rats suggest Stabilin-1 and -2 are broadly similar in young and old animals (Simon-Santamaria et al., 2010). Nevertheless, decline in fenestration with age can reduce clearance of drugs such as paracetamol (Mitchell et al., 2011). Similarly, clearance of gut derived LPS is impaired in cirrhosis due to reduced sinusoidal permeability leading to hyperactivation of plasma cells and increased immunoglobulin production (Liu et al., 2015). Capillarization of LSEC also restrict access to hepatocyte FcRn which normally transports antibody across epithelial barriers and maintains circulating antibody concentration (Yeung et al., 2009). Mice that are deficient in FcRn have reduced half-life of administered antibodies (Israel et al., 1996). Coupled with reduced expression of scavenger receptors such as DC-SIGN and FcγR on diseased LSEC this could profoundly alter antibody clearance kinetics. Similarly, occupancy of DC-SIGN by ligands such as viral and bacterial antigens (Gupta and Gupta, 2012) during infection could alter availability for binding antibody-based therapies. In situations of hepatic autoimmunity or disease, clearance of autoantibodies could be managed using FcRn blockers to enhance IgG degradation to manage autoantibodies or control clearance of therapeutic immunoglobulins (Vaccaro et al., 2005). Alternately specific engineering of monoclonal or bispecific antibodies to modify interactions with FcRn could also be used to improve pharmacokinetics (Schutten et al., 1993; Datta-Mannan et al., 2007; Lucas et al., 2018; Datta-Mannan, 2019). This may be particularly important in the context of treating chronic disease if an antibody-based therapy needs to be maintained at therapeutic levels for a long time. Indeed, anti-FcγRIIb antibodies have been suggested as a strategy to reduce

clearance of therapeutic antibodies for prolonged administration. However, these were rapidly cleared from the circulation since FcγRIIb is rapidly internalized once antibody binds (Williams et al., 2013). Nevertheless, it is clear that new approaches to antibody design are increasing our abilities to control the pharmacokinetics and targeting of therapeutic antibodies to maximize efficacy whilst minimizing off target effects. In conclusion we have highlighted the often-underestimated role of the liver sinusoidal endothelial cell to antibody clearance. We have also suggested how understanding the changing nature of LSEC in health and disease may explain variations in pharmacokinetics and toxicity in different populations and preclinical models. Challenges to antibody discovery programs are summarized in **Table 1**. Thus, it seems vital to ensure that future drug development pathways incorporate testing in models with truly representative features and cellular constituents to address issues of poor kinetics, unexpected toxicity and poor predictive ability.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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