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REVIEW

The Hepatic Lymphatic Vascular System: Structure, Function, Markers, and Lymphangiogenesis

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SUMMARY

Research on the lymphatic vascular system has advanced rapidly during the last decade, and lymphatic dysfunction is now implicated in the pathogenesis of multiple diseases. This review provides an overview of the lymphatic vascular system in the liver.

The lymphatic vascular system has been minimally explored in the liver despite its essential functions including maintenance of tissue fluid homeostasis. The discovery of specific markers for lymphatic endothelial cells has advanced the study of lymphatics by methods including imaging, cell isolation, and transgenic animal models and has resulted in rapid progress in lymphatic vascular research during the last decade. These studies have yielded concrete evidence that lymphatic vessel dysfunction plays an important role in the pathogenesis of many diseases. This article reviews the current knowledge of the structure, function, and markers of the hepatic lymphatic vascular system as well as factors associated with hepatic lymphangiogenesis and compares liver lymphatics with those in other tissues. *(Cell Mol Gastroenterol Hepatol 2016;2:733–749; http://dx.doi.org/10.1016/j.jcmgh.2016.09.002)*

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The lymphatic and blood vascular systems together constitute the circulatory system, and both have essential physiological activities. The lymphatic vascular system maintains tissue fluid homeostasis by collecting excess tissue fluid and returning it to the venous circulation. It also plays an essential role in the absorption and transport of dietary fat. Furthermore, lymphatics serve as the main conduits of antigens and antigen-presenting cells from the periphery to lymph nodes and are thus crucial for immune surveillance and acquired immunity.[1–4]

Lymphatic vascular research was impeded by a lack of knowledge about the markers and signaling pathways specific to the lymphatic vasculature. From 1995 to 1997, however, it was shown that vascular endothelial growth factor receptor (VEGFR)-3 is expressed in the lymphatic endothelium and that its ligand vascular endothelial growth factor (VEGF)-C promotes lymphangiogenesis.[5,6] This finding identifying signaling pathways specific to the lymphatic vasculature and subsequent discoveries of other specific markers for lymphatic endothelial cells (LyECs), such as lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1),[7] prospero homeobox protein 1 (Prox1),[8] and podoplanin,[9] significantly advanced lymphatic vascular research. As a consequence, it is now recognized that lymphatic vessel dysfunction plays an important role in the pathogenesis of various diseases.

However, in the liver, the lymphatic vascular system has been little explored. This review will provide an overview of the structure, function, and markers of the lymphatic vascular system as well as factors associated with lymphangiogenesis in the liver, highlighting both new findings and areas needing further study.

Structure of the Hepatic Lymphatic Vascular System

This section will address the structure of the lymphatic vascular system in general, followed by structural features specific to the liver. A detailed description of the anatomic structure of the lymphatic and hepatic lymphatic vascular systems is available in other review articles.[3,10–12]

Anatomy of the Lymphatic Vascular System

Lymphatic capillaries. Lymphatic fluid originates from plasma components leaked from blood capillaries into the interstitium and then enters lymphatic capillaries, which are blind-ended, thin-walled vessels consisting of a single layer of LyECs. Lymphatic capillaries are not covered by pericytes or smooth muscle cells and lack basement membranes.[13,14] They are highly permeable, with discontinuous "button-like" junctions through which interstitial fluid, macromolecules, and immune cells can be transported.[15] LyECs have anchoring filaments that are mainly composed of emilin-1 and fibrillin and bind LyECs to the surrounding extracellular matrix.[14,16,17] These filaments keep lymphatic vessel

Abbreviations used in this paper: CCl_4, carbon tetrachloride; EHE, epithelioid hemangioendothelioma; HA, hyaluronan; HBx Ag, hepatitis B x antigen; HCC, hepatocellular carcinoma; IFN, interferon; IL, interleukin; LSEC, liver sinusoidal endothelial cell; LyEC, lymphatic endothelial cell; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; mTOR, mammalian target of rapamycin; NO, nitric oxide; Prox1, prospero homeobox protein 1; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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lumens open, facilitating fluid intake in conditions of tissue swelling.

Collecting vessels. Lymphatic capillaries coalesce into collecting vessels, which are covered with smooth muscle cells and have basement membranes.[14] Collecting vessels lack the discontinuous junctions typical of lymphatic capillaries and are thus much less permeable. Collecting vessels can be divided into smaller functional units called lymphangions that have unidirectional bicuspid valves at each end.[18] The phasic contraction of smooth muscle cells covering lymphangions enables collecting vessels to act as pumps to drive lymphatic flow. Stimulation of smooth muscle cells causes depolarization of cell membrane and opens Ca^{2+} channels, resulting in Ca^{2+} influx and smooth muscle cell contraction. Smooth muscle cells also have stretch-activated Ca^{2+} channels that facilitate phasic contraction.[19,20] On the other hand, LyECs produce the vasodilator nitric oxide (NO) in response to shear stress from fluid flow, counteracting Ca^{2+}-dependent contraction.[21,22] Spatiotemporal alterations of Ca^{2+} and NO levels are thereby believed to modulate the phasic contraction of lymphangions.[23]

Lymph nodes and lymph trunks. Collecting vessels connect to 1 or more lymph nodes. Antigen-presenting cells including dendritic cells and macrophages in lymphatic fluid interact with lymphocytes in lymph nodes, facilitating adaptive immune responses. After reaching primary lymph nodes, lymphatic fluid flows to secondary central lymph nodes, tertiary central lymph nodes, and finally lymph trunks.[24] Lymphatic fluid from the left side of the body, abdomen, and lower limb ultimately drains into the thoracic duct, the largest lymphatic vessel, which is connected to the left subclavian vein (Figure 1), whereas lymphatic fluid from other

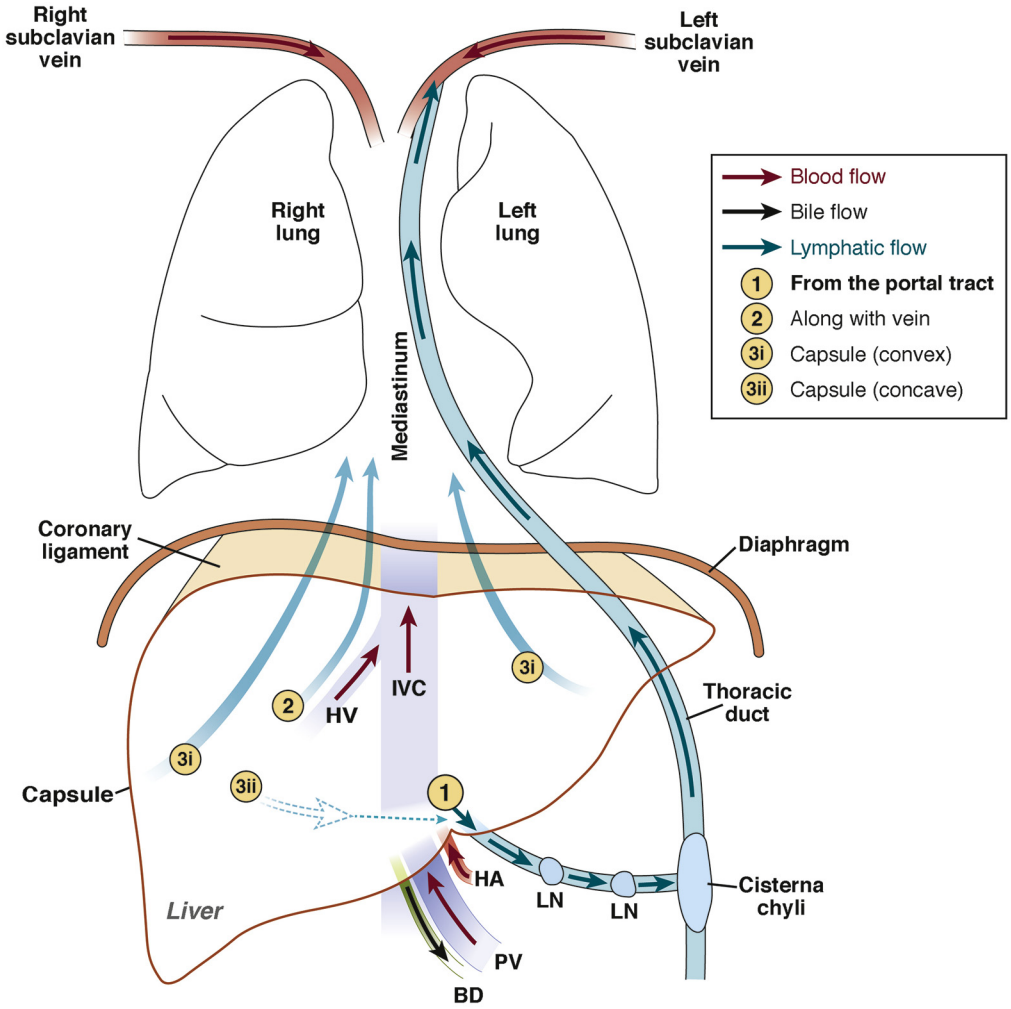

Figure 1. Schematic diagram of macro-anatomy of hepatic lymphatic vascular system. (*1*) Lymphatic capillaries in the portal tract coalesce into collecting vessels, which drain to lymph nodes at the hepatic hilum and the lesser omentum. Efferent lymphatic vessels (LV) from these lymph nodes connect to celiac lymph nodes, which drain to the cisterna chyli, the enlarged origin of the thoracic duct. Lymphatic fluid through the thoracic duct drains to the left subclavicular vein and returns to the systemic blood circulation. (*2*) Lymphatic vessels along the central vein (CV) converge into large lymphatic vessels along the hepatic vein (HV), which then traverse along the inferior vena cava (IVC) through the diaphragm toward mediastinal lymph nodes. (*3*) Lymphatic fluid running underneath the capsule of the convex surface of the liver (*3i*) drains to mediastinal lymph nodes through the coronary ligament, whereas that of the concave surface (*3ii*) drains to lymph nodes of the hepatic hilum and regional lymph nodes. BD, bile duct; HA, hepatic artery; LN, lymph node; PV, portal vein.

parts of the body drains into the right lymph trunk, which is connected to the right subclavian vein.[25] Lymphatic fluid that enters the subclavian veins returns to the systemic blood circulation.

Anatomy of the Hepatic Lymphatic Vascular System

A schematic diagram of the hepatic lymphatic system is shown in Figures 1 and 2. Unlike other tissues, the liver has sinusoids instead of capillaries.[26] Sinusoids, similar to lymphatic capillaries, are distinct from blood capillaries in that they consist of 1 layer of liver sinusoidal endothelial cells (LSECs) and lack basement membranes. Hepatic lymphatic fluid is thought to originate from plasma components filtered through the fenestrae of LSECs into the space of Disse, the interstitial space between LSECs and hepatocytes.[10,11] Fluid in the space of Disse primarily flows through the space of Mall, a space between the stroma of the portal tract and the outermost hepatocytes,[27] into the interstitium of the portal tract and then into lymphatic capillaries. Some portion of the fluid in the space of Disse flows into the interstitium around the central vein, which is located in the center of the liver acinus and connected to the hepatic vein,[28] or underneath the hepatic capsule (Figure 2).

Lymphatic capillaries in the portal tract coalesce into collecting vessels and drain to lymph nodes at the hepatic hilum, whereas lymphatic vessels along the central vein converge into 5–6 large lymphatic vessels that traverse along the inferior vena cava through the diaphragm toward posterior mediastinal lymph nodes. Lymphatic fluid running underneath the capsule of the convex surface of the liver drains to mediastinal lymph nodes through the coronary ligament, whereas that fluid running along the concave surface drains to lymph nodes in the hepatic hilum and to regional lymph nodes (Figure 1).[10–12,29] On the basis of their locations, lymphatic vessels along the portal tract and the central vein are called the deep lymphatic system, and those along the hepatic capsule are called the superficial lymphatic system.[10–12,29]

Markers of Lymphatic Vessels

Lymphatic vessel markers generally refer to those specific to LyECs. The markers LYVE-1,[7,30,31] podoplanin,[9,32] Prox1,[8,33–35] and VEGFR-3[5] are most commonly used for microscopic imaging of lymphatic vessels.[36] Identification of more specific markers for the liver is needed because the most common LyEC markers, LYVE-1 and Prox1, are also expressed in LSECs and hepatocytes, respectively. Table 1 summarizes LyEC markers histologically examined in the liver.

Lymphatic vessel endothelial hyaluronan receptor 1. LYVE-1 is a lymphatic vessel endothelial hyaluronan (HA) receptor, a homolog of the CD44 HA receptor,[7] that belongs to the superfamily of Link proteins containing a conserved HA-binding domain known as the Link module.[37]

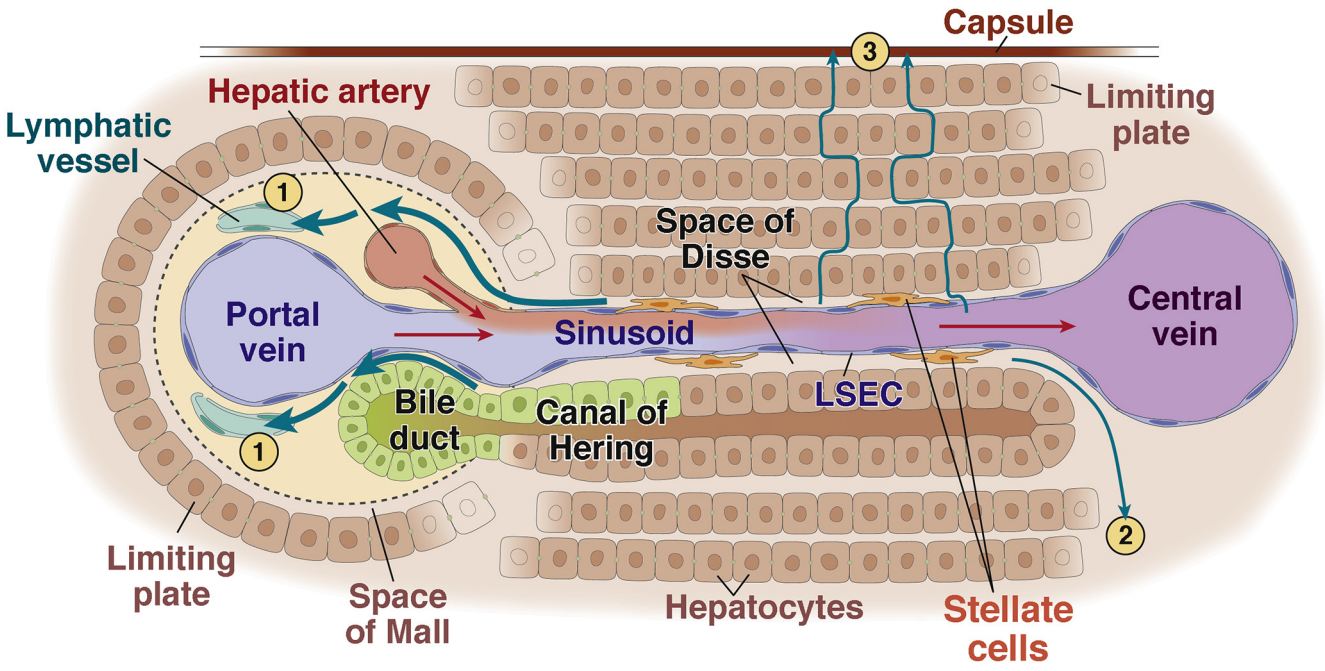

Figure 2. Schematic diagram of the micro-anatomy of the hepatic lymphatic vascular system. Blood flow (*red arrows*) from the portal vein (PV) and hepatic artery (HA) enters the liver. Plasma components are filtered through LSECs into the space of Disse, the interstitial space between LSECs and hepatocytes, and are regarded as the source of lymphatic fluid. Lymphatic fluid in the space of Disse mostly flows through the space of Mall, the space between the stroma of the portal tract and the outermost hepatocytes, into the interstitium of the portal tract and then into lymphatic capillaries (*1*). Some portion of the lymphatic fluid in the space of Disse flows into the interstitium around the central vein (*2*) or underneath the hepatic capsule (*3*).

Table 1. Lymphatic Markers

Marker	Postnatal expression except for lymphatic vessels		Hepatic expression in pathologic conditions	Reference
	Liver	Other organs/cells		
LYVE-1	Sinusoidal endothelial cells	A portion of macrophages, pulmonary capillaries, epididymal adipose tissue, mesentery, eye (cornea, sclera, choroid, iris, and retina), wounded skin, and malignant tumors (melanoma and insulinoma)	In chronic hepatitis and liver cirrhosis in humans, LYVE-1(+) lymphatic vessels increase, but LYVE-1(+) sinusoidal endothelial cells decrease.	38, 40–43, 105, 159–164
Prox1	Hepatocytes	Adrenal medulla, megakaryocytes, and platelets	Intrahepatic CCC, ductular cells in cirrhotic livers, and HCC in humans.	8, 52, 58, 59
Podoplanin	Cholangiocytes	Inflammatory macrophages, mesothelial cells, cardiomyocytes, FRCs, follicular dendritic cells, TH17 cells, and osteoblasts	Podoplanin(+) lymphatic vessels increase in decompensated cirrhosis in humans. Podoplanin(+) FRCs increase in livers of primary biliary cirrhosis patients. EHE and angiomyolipoma in humans.	32,72, 75–79, 165, 166
VEGFR-3	Cholangiocytes	A portion of macrophages, proliferating blood vessels, and fenestrated capillaries in endocrine glands, choroid plexus, kidney, and small intestine	HBx Ag–positive HCC and hepatic progenitor cells in primary biliary cirrhosis in humans.	80, 83–85, 102, 167, 168
CCL21	Sinusoidal endothelial cells	A portion of dendritic cells, HEVs of lymph nodes and Peyer's patches, T-cell areas of spleen, lymph nodes, and Peyer's patches	Lymphoid tissue in primary biliary cirrhosis and primary sclerosing cholangitis in humans.	169–171
MMR1	Sinusoidal endothelial cells and Kupffer cells	A portion of macrophages, sinusoidal endothelial cells in bone marrow and spleen, perivascular microglia, and glomerular mesangial cells	Unknown	172–175
Desmoplakin	Basolateral plasma membrane of hepatocytes and cholangiocytes	Esophagus, intestine, colon, salivary gland, mammary gland, sweat gland, thymus, and endocervix	Entire plasma membrane of HCC cells	174, 176–179
Integrin α_9	Hepatocytes	Airway epithelial cells, keratinocytes, muscle cells (smooth/skeletal/cardiac), neutrophils, osteoclasts, and oocytes	Unknown	174, 180

CCC, cholangiocellular carcinoma; CCL21, C-C motif chemokine ligand 21; FRC, fibroblastic reticular cell; HEV, high endothelial venules; MMR, macrophage mannose receptor 1.

Its structural features suggest that LYVE-1 may be involved in the transport of HA across the lymphatic endothelium. LYVE-1 is strongly expressed on the entire luminal and abluminal surfaces of LyECs, even on the fine filopodia of growing vessels during lymphangiogenesis.

No definite alterations in lymphatic vessel structure and function were reported for LYVE-$1^{-/-}$ mice.[38] However, diphtheria toxin-induced LYVE-1 depletion in mice caused acute loss of lymphatic lacteals in intestinal villi and lymphatic vessels in systemic lymph nodes. These changes resulted in the structural distortion of blood capillaries and villi, leading to death due to sepsis within 60 hours after LYVE-1 depletion.[39] These observations indicate that LYVE-1 plays an important role in the maintenance of the lymphatic vascular system, especially lacteals in intestinal villi and lymph nodes. Compensatory mechanisms in the setting of congenital loss of LYVE-1 may explain the relatively mild phenotype of these mice.

In the liver, LYVE-1 is expressed not only in LyECs but also in LSECs, as shown in mice[40] and humans.[40–43] However, LYVE-1 positivity in LSECs was reported to diminish in inflamed human livers such as those of chronic hepatitis and cirrhosis.[40,42] Expression levels of LYVE-1 in human hepatocellular carcinoma (HCC) negatively correlated with the overall survival of patients.[44]

Prospero homeobox protein 1. Prox1, a homolog of the *Drosophila melanogaster* homeobox gene prospero, is a transcription factor and regulates genes related to LyECs, including VEGFR-3 and podoplanin.[45,46] Prox1 is essential for the development of the lymphatic vascular system[8] and also plays a role in the development of other tissues, including the lens,[34,47] retina,[48] heart,[49] central nervous system,[50] pancreas,[51] and liver.[52,53] Prox1 is expressed in the nucleus in contrast to other lymphatic markers that are expressed in the cytoplasm or on the plasma membrane.

Prox1 is essential for budding of lymphatic endothelial sacs[8]; Prox$1^{-/-}$ mice lack a lymphatic vascular system and die at approximately E14.5. Prox1 heterozygote mice die a few days after their birth and demonstrate dysfunction of lymphatic vessels with chylous ascites.[8,33,47] Several lines of Prox1 promoter-directed reporter mice have recently been established as research tools (GFP,[54] mOrange,[55] and tdTomato[56–58]).

In the early endoderm, Prox1 expression is restricted to the primordia of the liver and pancreas.[51] Prox1 regulates hepatocyte migration during liver morphogenesis[51] and is expressed in postnatal hepatocytes, although not in postnatal pancreas.[52] In humans, cholangiocytes of normal livers were negative for Prox1 expression, but intrahepatic cholangiocarcinoma and ductular cells in fibrotic septa of cirrhotic livers and HCC were positive.[59] In addition, expression levels of Prox1 (like LYVE1) in human HCC negatively correlated with the overall survival of patients.[60] Prox1 acts together with nuclear receptors, such as hepatocyte nuclear factor 4α,[61] estrogen-related receptor α,[62,63] liver receptor homolog-1,[64] and retinoic acid-related orphan receptors α/γ,[65] and regulates bile acid synthesis[64] and circadian metabolism in the liver.[63,65]

Podoplanin. Podoplanin is a type I transmembrane glycoprotein essential for the development of the heart,[66–69] lung,[70] spleen, and lymph nodes.[71] Its expression is regulated by Prox1.[45] Podoplanin is also a ligand of C-type lectin receptor CLEC-2, which is highly expressed in platelets and immune cells and promotes platelet aggregation and activation.[72]

Podoplanin$^{-/-}$ mice die at birth as a result of respiratory failure. These mice have congenital lymphedema caused by lymphatic vessel defects, although blood vessel formation is normal.[32] Podoplanin heterozygote mice are healthy and fertile, with a partial incomplete lymphatic vessel network.[32] Keratinocyte-specific podoplanin-deficient mice[73] and a tamoxifen-inducible podoplanin depletion mouse model (Pdpn$^{f/f}$, CagCre)[74] have recently been developed.

Histologic analysis of normal mouse livers showed expression of podoplanin in cholangiocytes in addition to LyECs.[75] In humans, podoplanin-positive lymphatic vessels were increased in the livers of patients with decompensated cirrhosis,[76] and podoplanin-positive fibroblastic reticular cells were increased in livers of patients with primary biliary cirrhosis.[77] Podoplanin has proven to be a useful histologic marker for diagnosing patients who have vascular tumors with lymphatic differentiation, such as epithelioid hemangioendotheliomas (EHEs)[78] and angiomyolipomas.[79]

Vascular endothelial growth factor receptor. VEGFR-3 is a membrane-anchored tyrosine kinase and the receptor for VEGF-C and VEGF-D. It plays a crucial role in lymphangiogenesis. In early embryogenesis before LyEC differentiation, VEGFR-3 is expressed in most endothelial cells, but in the later stages of development, its expression becomes mostly restricted to the lymphatic endothelium.[5]

VEGFR-$3^{-/-}$ mice have lymphatic vessel defects and die at approximately E10.5,[80] whereas VEGFR-3 heterozygous mice present with leaky lymphatic vessels and transient chylous ascites.[80,81] A mouse line (*Vegfr3*^{EGFPLuc}) in which a dual reporter for fluorescence and luminescence is expressed under VEGFR-3-promoter was established recently, enabling luminescence imaging of tumor-induced lymphangiogenesis.[82]

VEGFR-3 is expressed by cholangiocytes in normal rat livers and is increased in cholestatic rat livers after bile duct ligation.[83] Hepatic progenitor cells were also found to express VEGFR-3 in patients with primary biliary cirrhosis.[84] Hepatitis B x antigen (HBx Ag), one of the antigens of hepatitis B virus, promotes hepatocarcinogenesis by upregulating expression of genes associated with proliferation of hepatocytes; upregulation of VEGFR-3 expression was observed in HBx Ag–positive human HCC, and the prognosis of patients with VEGFR-3–positive HCC was worse than for those with VEGFR-3–negative HCC.[85]

Lymphangiogenesis

This section addresses the mechanism of lymphangiogenesis in the postnatal stage and factors that affect lymphangiogenesis, including inflammatory cells, in the lymphatic system in general and then summarizes the

implications of lymphangiogenesis in the pathophysiology of liver diseases.

Factors Associated With Lymphangiogenesis

In the postnatal stage, lymphatic vessels are mostly quiescent, and lymphangiogenesis generally occurs in pathologic conditions such as tissue repair, inflammation, and tumor-related conditions.[86] Many cytokines and growth factors have been reported to promote lymphangiogenesis or inhibit lymphangiogenesis, as summarized in Table 2. The extent and duration of lymphangiogenesis are determined by the balance between pro- and anti- lymphangiogenic factors.[87,88]

Intracellular Signaling Pathways in Lymphangiogenesis

Signaling pathways in lymphangiogenesis have largely been determined in studies of developmental lymphangiogenesis. Signaling via VEGF-C/D and VEGFR-3 is the most well-known pathway for lymphangiogenesis (Figure 3).[6] VEGF-C or VEGF-D binding to VEGFR-3 results in autophosphorylation of multiple C-terminal tyrosine residues in VEGFR-3,[89] which transduces signals through the Ras/Raf/MEK/ERK pathway.[90] Signal transduction also occurs through the PI3K/Akt pathway,[91] which causes phosphorylation of Akt, thereby activating mammalian target of rapamycin (mTOR) and Rac1.[92] Activation of these signaling pathways facilitates LyEC proliferation and migration, ie, lymphangiogenesis.[91] Chronic inflammation and malignant tumors in the liver induce several pro-lymphangiogenic growth factors including VEGF-C/D. However, a direct link between these increased pro-lymphangiogenic growth factors and lymphangiogenesis in these pathologic conditions remains to be demonstrated (Figure 3). Excellent review articles are available detailing signaling pathways in lymphangiogenesis.[93–95]

Role of Immune Cells

Adaptive immune responses are initiated by the migration of immune cells to inflamed sites where they phagocytose pathogens and transmigrate through lymphatic vessels to lymph nodes to present antigens to T cells. However, immune cells not only migrate through lymphatic vessels but also interact with lymphatic vessels and promote lymphangiogenesis.[96] An increase in lymphatic vessels helps infiltrating immune cells exit inflamed sites via lymphatic vessels and accelerates resolution of inflammation.[97–99]

Macrophages. Among the various immune cells, macrophages interact most with lymphatic vessels. LyECs secrete chemotactic factors, such as C10, monocyte chemoattractant protein-1, and macrophage inflammatory protein-1, to attract macrophages.[100] Macrophages in turn secrete lymphangiogenic cytokines such as VEGF-C, VEGF-D, and VEGF-A,[101] which promote tumor-associated lymphangiogenesis[102] and inflammation-induced lymphangiogenesis, as shown in the cornea,[103] skin,[98] and tail.[104] Macrophages were recently suggested to have the ability to transdifferentiate to LyECs.[105–107] However, this is controversial and requires further investigation.

Dendritic cells. Upregulation of inflammatory cytokines such as tumor necrosis factor-α and interleukin (IL) 1β in inflamed tissues promotes expression of chemokines (eg, CCL21/CCL19 and CXCL12) and their receptors (eg, CCR7 and CXCR-4) in LyECs and dendritic cells,[108–111] which enhances transmigration of dendritic cells through LyECs.[112,113] Inflammatory cytokines also increase expression of adhesion molecules such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and E-selectin in LyECs and promote dendritic cell transmigration to lymphatic vessels.[114] Dendritic cells have also been reported to secrete VEGF-C and promote lymphangiogenesis.[115]

T cells. In a mouse model of tail lymphedema, nude mice exhibited less edema than wild-type mice, concomitant with decreased lymphangiogenic cytokines and increased anti-lymphangiogenic cytokines. The balance between these cytokines was modulated by T-cell–mediated inflammation.[88] T cells negatively regulated lymph node lymphangiogenesis by secreting interferon (IFN)-γ in mice.[116]

B cells. B cells promote lymphangiogenesis in inflamed lymph nodes by secreting a robust amount of VEGF-A in mice given keyhole-limpet hemocyanin emulsified in complete Freund's adjuvant (an experimental model of inflamed lymph nodes).[117] Interestingly, VEGF-C was not detected in this study. Another study that used transgenic mice overexpressing VEGF-A specifically in B cells showed increased lymphangiogenesis as well as angiogenesis.[118]

Neutrophils. Neutrophils are reported to contribute to lymphangiogenesis by modulating the bioavailability and bioactivity of VEGF-A and by secreting VEGF-D.[119] The bioavailability of VEGF-A is increased by the secretion of matrix metalloproteinases 9 and heparanase. Depletion of neutrophils in mice resulted in skin inflammation in response to immunization or contact hypersensitization, and lymphangiogenesis was decreased in these mice with increased local inflammation, suggesting that neutrophils play a role in lymphangiogenesis and that lymphangiogenesis is helpful for reducing inflammation.

Lymphangiogenesis in the Liver

Because 25%–50% of lymph passing through the thoracic duct originates in the liver,[1,120] the liver can be considered the most important organ for lymphatic fluid production. However, the lymphatic vascular system in the liver has been minimally explored. A small number of studies have reported on hepatic lymphangiogenesis in pathologic conditions such as chronic hepatitis, liver fibrosis/cirrhosis, portal hypertension, malignant tumors, and post-transplantation. This section summarizes these studies.

Chronic hepatitis, liver fibrosis, and cirrhosis. Resistance to sinusoidal blood flow increases in cirrhotic livers because of architectural deformations including around the portal and central venules. Consequently, sinusoidal hydrostatic pressure is elevated, and plasma components filtrated through sinusoids (which form

Table 2. Lymphangiogenic and Anti-lymphangiogenic Factors

	Experimental model	Remarks	Reference
Lymphangiogenic factors			
VEGF-A	Mouse corneal lymphangiogenesis	VEGF-A recruits macrophages, which promote lymphangiogenesis by secreting VEGF-C/VEGF-D.	103
	Mouse subcutaneous immunization model	VEGF-A expression is upregulated concomitantly with lymphangiogenesis in LNs of immunized mice.	117
	Oxazolone sensitized delayed-type hypersensitivity in mouse ear	Systemic blockade of VEGF-A attenuates lymphangiogenesis in draining LNs.	181
	HSV-1 infection of cornea	HSV-1 causes lymphangiogenesis by promoting infected cells to secrete VEGF-A.	182
VEGF-C, VEGF-D	VEGF-C transgenic mouse	VEGF-C promotes LyEC proliferation and LV enlargement in the skin.	6
	Isolated LyEC	VEGF-C stimulates survival, growth, and migration of LyEC.	91
	FGF-2–induced corneal lymphangiogenesis	VEGFR-3 blockade cancels lymphangiogenesis.	183
	Chronic airway inflammation	VEGFR-3 blockade cancels lymphangiogenesis.	184
	LPS-induced peritonitis	VEGF-C and VEGF-D promote lymphangiogenesis in diaphragm.	185
Ang 2	Mouse corneal lymphangiogenesis	Ang 2 is upregulated in inflamed cornea, and Ang2 blockade inhibits inflammatory lymphangiogenesis.	186
	Mouse corneal lymphangiogenesis	Ang 2 is expressed in lymphatic vessels and macrophages in inflamed cornea. Inflammatory lymphangiogenesis of cornea is suppressed in Ang2 knockout mice. Ang2 blockade inhibits LyEC proliferation and capillary tube formation.	187
HGF	Canine primary LyEC, rat tail lymphedema	HGF promotes proliferation and migration of LyEC. Weekly HGF gene transfer improves lymphedema in vivo.	188
LT	CCL21 transgenic mouse, RAG knockout mouse defective in T and B cell	LT overexpression by CCL21 transgene promotes lymphangiogenesis in thyroid. T-cell depletion cancels this phenomenon.	189
	LTα knockout mouse, LTα transgenic mouse	LTα gene deletion decreases LV. Ectopic LTα expression causes lymphangiogenesis in tertiary lymphoid organs.	190
IL1β	Mouse corneal lymphangiogenesis	IL1β promotes lymphangiogenesis by upregulating expression of VEGF-A, VEGF-C, and VEGF-D.	191
IL7	Breast cancer cell lines, subcutaneous injection of Matrigel and/or IL7 and/or breast cancer cell lines	IL7 promotes VEGF-D expression of cell lines in vitro and promotes lymphangiogenesis in vivo.	192
	HECV cell line (originated from human umbilical cord), subcutaneous injection of Matrigel and/or IL7 and/or HECV cell	IL7 promotes expression of Prox1, LYVE-1, and podoplanin and proliferation, migration, and tubular formation of LyEC via upregulation of VEGF-D.	193
IL8	Human primary LyEC, IL8 transgenic mouse and Prox1-GFP mouse	IL8 promotes proliferation, migration, and tube formation of LyEC. IL8 overexpression promotes lymphangiogenesis in vivo.	194

Table 2. Continued

	Experimental model	Remarks	Reference
IL17	Cornea micro pocket assay, autoimmune ocular disease mouse	IL17 promotes proliferation of LyEC via upregulation of VEGF-D. Blockade of IL17 decreases corneal lymphangiogenesis.	195
IL20	Human telomerase-transfected dermal LyEC	IL20 promotes proliferation, migration, and tubular formation of LyEC via PI3K and mTOR pathways.	196
Anti-lymphangiogenic factors			
TGF-β	Human dermal lymphatic microvascular endothelial cells	TGF-β inhibits LyEC proliferation, cord formation, migration, expression of lymphatic markers (LYVE-1, Prox1), and lymphangiogenesis by VEGF-A/C via TGF-β type I receptor.	197
	Mouse tail skin excision and lymphatic vessel ligation	TGF-β1 inhibition promotes lymphatic vessel regeneration. TGF-β1 inhibits LyEC proliferation and fibrosis.	198
	Biopsy specimens from limbs of secondary lymphedema patients and mouse tail skin excision	TGF-β1 positive cells increase 3-fold in human lymphedema specimens. TGF-β1 inhibition decreases fibrosis, increases lymphangiogenesis and lymphatic function.	199
BMP2	Zebrafish BMP2 transgenic model	BMP2 inhibits LyEC differentiation from cardinal veins via inhibition of Prox1 expression.	200
IFN-α, IFN-γ	LyEC isolated from pig thoracic duct	IFN-α or IFN-γ decreases LyEC proliferation and migration. Treatment with both IFN-α and IFN-γ promotes LyEC apoptosis.	201
	Cervical LNs of T-cell–deprived mouse	T cells inhibit lymphangiogenesis in LNs by secreting IFN-γ.	116
IL4, IL13	Mouse LyEC isolated from LNs, human dermal LyEC, mouse asthma model	IL4 and IL13 inhibit expression of Prox1 and LYVE-1 and tube formation of LyEC. Blockade of IL4 and/or IL13 increases the density and function of lung LVs in asthma model.	202
IL27	Human dermal lymphatic microvascular endothelial cells	IL27 inhibits LyEC proliferation and migration via STAT1/CXCL10, CXCL-11 axis.	203
Activin A	Subcutaneous injection of melanoma cell line to mouse	Activin A reduces lymphangiogenesis in melanoma model and inhibits sprouting of LyEC via phosphorylation of SMAD2.	204

FGF-2, fibroblast growth factors-2; HGF, hepatocyte growth factor; HSV-1, herpes simplex virus 1; LN, lymph node; LPS, lipopolysaccharide; LT, lymphotoxin; LV, lymphatic vessel; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; STAT, signal transducer and activator of transcription; TGF, tumor growth factor.

lymphatic fluid) increase. In cirrhotic patients, lymphatic fluid produced in the liver increases up to 30-fold,[121–125] and liver surface lymphatic vessels dilate, as shown by peritoneoscopic observation.[126]

Ascites formation in association with cirrhosis is one of the most recognized clinical manifestations of lymphatic vascular disorders. How ascites is formed still remains to be elucidated. Although several theories have been put forward,[127–129] the most accepted currently is "the peripheral arterial vasodilation theory", also known as "the forward theory".[130–132] According to this theory, splanchnic arterial vasodilation caused by portal hypertension results in underfilling of the splanchnic arterial circulation (hypovolemia). In moderate stages, the hypovolemia is compensated for by renal retention of sodium and water. However, in severe portal hypertension with splanchnic arterial vasodilation, sodium and water retention is persistent and leads to leakage of fluid into the peritoneal cavity. When its amount exceeds the absorption capacity of lymphatic vessels, ascites results.[129,133]

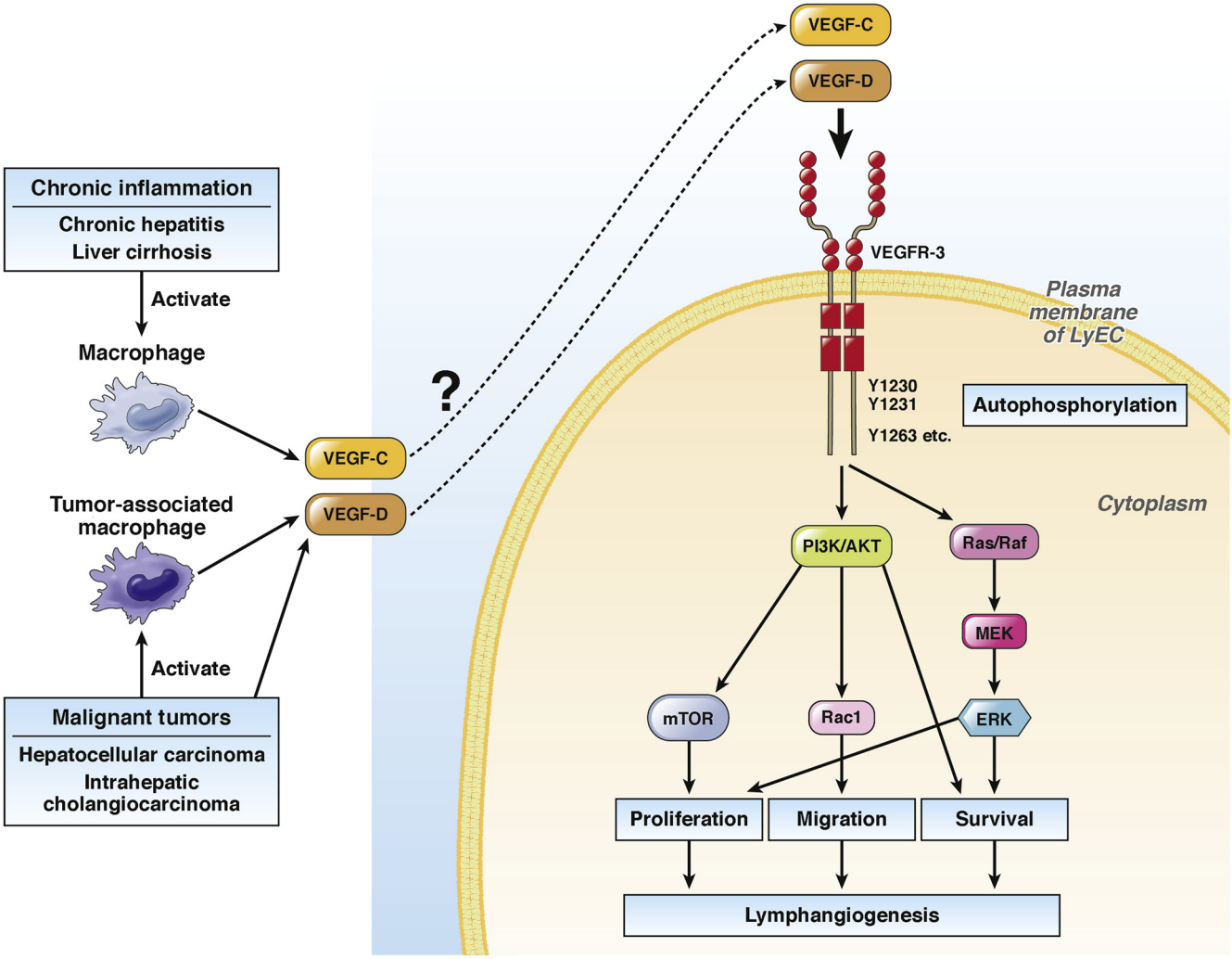

Figure 3. Intracellular signaling pathways in lymphangiogenesis. Signaling via VEGF-C/D and VEGFR-3 is the most well-known pathway for lymphangiogenesis. VEGF-C or VEGF-D binds to its receptor VEGFR-3 in the plasma membrane of LyECs, which facilitates signal transduction through various intracellular signaling pathways, leading to lymphangiogenesis. In the liver, activated macrophages in chronic inflammatory conditions, such as chronic hepatitis and liver cirrhosis, secrete VEGF-C and/or VEGF-D. Malignant liver tumors, such as HCC and intrahepatic cholangiocarcinoma, also secrete VEGF-C and/or VEGF-D. Furthermore, these malignant tumors activate tumor-associated macrophages, which also secrete VEGF-C and/or VEGF-D. Secreted VEGF-C and VEGF-D are likely related to lymphangiogenesis in liver diseases through VEGFR-3–mediated pathways.

On a related note, impaired lymphatic drainage in the splanchnic and peripheral regions was reported in cirrhotic rats with ascites. This was accompanied by increased activity of endothelial NO synthase and production of NO by LyECs in these regions.[134] In addition, smooth muscle cell coverage of lymphatic vessels in these regions was significantly decreased. Treatment of these cirrhotic rats with an NO synthase inhibitor improved lymphatic drainage, decreased ascites volume, and increased smooth muscle cell coverage. This study thus demonstrates a role for NO in the impairment of lymphatic vessels in splanchnic and peripheral regions and in the development of ascites. It is not known whether lymphatic vessels in human cirrhotic livers show similar pathologic features.

The occurrence of hepatic lymphangiogenesis was reported for the first time in liver fibrosis and cirrhosis by Vollmar et al[135] in 1997. They found lymphatic vessels to be increased and enlarged in rat liver cirrhosis induced by carbon tetrachloride (CCl_4). These observations were confirmed the following year in patients with chronic viral hepatitis/cirrhosis.[136]

Microarray analysis demonstrated a 4-fold increase in VEGF-D expression in endothelial cells from CCl_4-induced cirrhotic rat livers as compared with control rat livers. Because VEGF-D is a well-known lymphangiogenic factor that binds to VEGFR-3,[137] which is also highly expressed in the LyECs of these cirrhotic rats,[5] increased VEGF-D could be associated with the lymphangiogenesis observed in liver cirrhosis (Figure 3).

Lymphangiogenesis also occurs in idiopathic portal hypertension in human patients.[138] It was presumed that increased lymph production that was due to increased

portal pressure caused lymphangiogenesis. In 2 rat models of portal hypertension (portacaval shunt and portal vein ligation), upregulation of *Vegfr-3* expression was observed, leading us to speculate the occurrence of lymphangiogenesis.[139] However, the significance and mechanism of hepatic lymphangiogenesis, including in chronic hepatitis and liver fibrosis and cirrhosis, remain unknown.

Malignant tumors. Lymphatic vessels play a pivotal role in the pathogenesis of malignant tumors by serving as a pathway through which tumor cells metastasize. The incidence of lymph node metastasis differs among tumors. For example, it is 5.1% in HCC and 45.1% in intrahepatic cholangiocarcinoma. The prognosis of tumor-bearing patients with lymph node metastasis is worse than in cases without such metastasis.[140,141] Many malignant tumors secrete lymphangiogenic factors such as VEGF-C and VEGF-D and promote lymphangiogenesis in their adjacent tissues, which helps tumor cells to metastasize to lymph nodes,[142] and many studies have demonstrated that tumor-associated macrophages play a vital role in lymphangiogenesis in malignant tumors by secreting VEGF-C and VEGF-D.[102,143–145] In intrahepatic cholangiocarcinoma, the lymphatic vessel density of surgically resected tumors was positively correlated with the incidence of lymphatic metastasis.[146] In HCC, VEGF-C expression was positively correlated with the size of tumors and the number of extrahepatic metastases and was negatively correlated with disease-free survival time.[147] Thus, blockade of VEGF-C may be a potential therapeutic strategy against malignant tumors. A VEGF-C neutralizing antibody (VGX-100) is now the subject of a Phase I clinical trial for adult patients with advanced or metastatic solid tumors (NCT01514123).[148]

Post-transplant lymphangiogenesis. In solid organ transplants, lymphatic vessel connections between the graft and the recipient are interrupted. Because lymphatic vessels are essential for adaptive immunity, the association between lymphangiogenesis and graft rejection has received considerable attention. Post-transplant lymphangiogenesis in grafts was associated with acute cellular graft rejection in transplants of various organs (kidney,[149–151] heart,[152] and lung[153]) in humans. However, the pathologic role of post-transplant lymphangiogenesis in graft rejection remains unclear.[151] Post-transplant lymphangiogenesis could be detrimental if newly formed lymphatic vessels promote antigen presentation in draining lymph nodes and provoke alloimmune responses that result in graft rejection. On the other hand, these newly formed lymphatic vessels could be beneficial if they efficiently clear immune cells. In a rat model of liver transplantation, post-transplant lymphangiogenesis in grafts was associated with long-term survival of recipients for more than 90 days.[154] In addition, rats that had failed grafting by 11 days with acute cellular rejection and antibody-mediated rejection showed disappearance of lymphatic vessels from severely rejected areas, suggesting that lymphatic vessels have an important role in mitigation of inflammation at least in the early stage of transplantation. Further investigations to determine the mechanism and the time course of clearance of infiltrating immune cells by lymphatic vessels, especially in the early post-transplant period, may help increase transplant success.

Conclusions and Perspective

The lymphatic vascular system has been poorly studied in the liver. To drive research in this area, it is essential to identify better markers for LyECs that do not overlap with markers for LSECs, hepatocytes, and other liver cells. The development of experimental models for studying the lymphatic vascular system in postnatal livers will be important in examining its role and molecular mechanisms in physiological and pathophysiological conditions. Although this field is wide open, it may be helpful to identify specific questions particularly in need of study.

First, the mechanism of hepatic lymphangiogenesis is largely unknown. The VEGF-C/VEGFR-3 axis is considered the most potent signaling pathway that regulates lymphangiogenesis in other organs.[95] However, cellular sources of VEGF-C and VEGFR-3 have not been fully identified in the liver. Furthermore, as shown in Table 2, many other molecules are reported to regulate lymphangiogenesis. These molecules are mostly observed in the liver in physiological and pathophysiological conditions. It would be worth characterizing these molecules in relation to hepatic lymphangiogenesis.

Second, although the relationship between the lymphatic vascular system and metastasis is well-known and the growth of lymphatic capillaries in liver tumors has been observed, the role of lymphatic capillary growth in the development and progression of liver tumors is largely unknown. As for angiogenesis, it would be interesting to investigate lymphangiogenesis in liver cancer.

Third, inflammation is closely related to the development of many liver diseases, and infiltrating immune cells are drained to lymphatic vessels. Thus, it would be interesting to examine lymphangiogenesis in relation to inflammation in the liver. It is also unknown how immune cells recognize lymphatic vessels at the time of migration. Elucidation of these mechanisms may help in the development of anti-inflammatory strategies that facilitate immune cell clearance.

Fourth, although LyECs are derived from cardinal veins[8,81] and LSECs are derived from the septum transversum,[155] LyECs and LSECs have many similarities. Both LyECs and LSECs express LYVE-1.[40–43] VAP-1, a type II transmembrane protein that supports leukocyte adhesion, and reelin, a glycoprotein that is associated with embryonic development, are also expressed in both LyECs and LSECs.[156,157] Furthermore, under normal conditions, neither LyECs nor LSECs are associated with basement membranes. Examining the similarities and differences between these 2 types of endothelial cells could help to understand endothelial cell–related liver function.

In summary, the lymphatic vascular system in the liver is a large open area for investigation.[158] More research will significantly advance our understanding of liver physiology and pathophysiology and in turn contribute to the

development of new therapeutic strategies for many liver diseases.

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