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The Role of Hepatic and Splanchnic Lymphatic System in Portal Hypertension and Ascites

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Abstract
Purpose of Review The lymphatic network plays a major role in maintaining tissue fluid homeostasis. Therefore, several pathological conditions associated with edema formation result in deficient lymphatic function. However, traditionally, the lymphatic system has been underestimated until recent years when it has been noticed the importance of this system in chronic liver disease. This review highlights the knowledge of lymphatic biology in the context of portal hypertension and liver cirrhosis.
Recent Findings Among different roles of lymphatic system in liver disease, two remarkable ones are the contribution in ascites accumulation and the hepatic lymphangiogenesis in portal hypertension which is regulated by sympathetic nerves.
Summary The identification of novel pathological mechanisms has focused efforts into correction of structural changes and function affecting lymphatic vessels in liver disease. Despite the knowledge gained, we still have to face many unresolved questions concerning the role played by the lymphatic system in chronic liver disease and the design of therapeutic targeting.

Keywords Lymphatic system · Chronic liver disease · Nitric oxide · Ascites · Portal hypertension

Introduction

The lymphatic vasculature, sometimes underestimated, is a remarkable part of the vascular system due to the significant and different functions that performs. The main function of lymphatic vessels is to collect fluid leaked from blood vessels, called the lymph—usually rich in proteins—and transport it back into bloodstream [1]. Lymphatic vessels also have an important immune surveillance function, as they import various antigens and activated antigen-presenting cells into the lymph nodes and export immune effector cells and humoral response factors into blood circulation [2]. Furthermore, lymphatic vessels are essential for absorbing long chain fatty acids and fat-soluble vitamins, as well as lipophilic compounds released into the intestine as chylomicrons [3]. Alterations in the functionality of lymphatic system may lead to a deficient immune response [4], accumulation of fats, and tissue inflammation by lymph accumulation (lymphedema) [5–7]. This review highlights the knowledge of lymphatic biology in the context of portal hypertension and liver cirrhosis and how the lymphatic system contributes to the pathogenesis of the disease and its role in ascites accumulation.

This article is part of the Topical Collection on *Portal Hypertension*

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Origin of Lymphatic Vasculature

During embryonic development, endothelial precursors form a primary plexus that results in the formation of blood vessels in a process known as vasculogenesis. As endothelial cells (ECs) start proliferating, the plexus expands and originates the vascular network (angiogenesis). This network undergoes maturation through recruitment of smooth muscle cells (SMCs) and vascular specialization becoming a highly organized functional blood system composed of arteries and veins. This differentiation process is regulated by different factors such as active Notch for arterial differentiation, Notch inhibition through Coup-TFII for venous differentiation and members of the vascular endothelial growth factor (VEGF) family and its receptors (Fig. 1) [8].

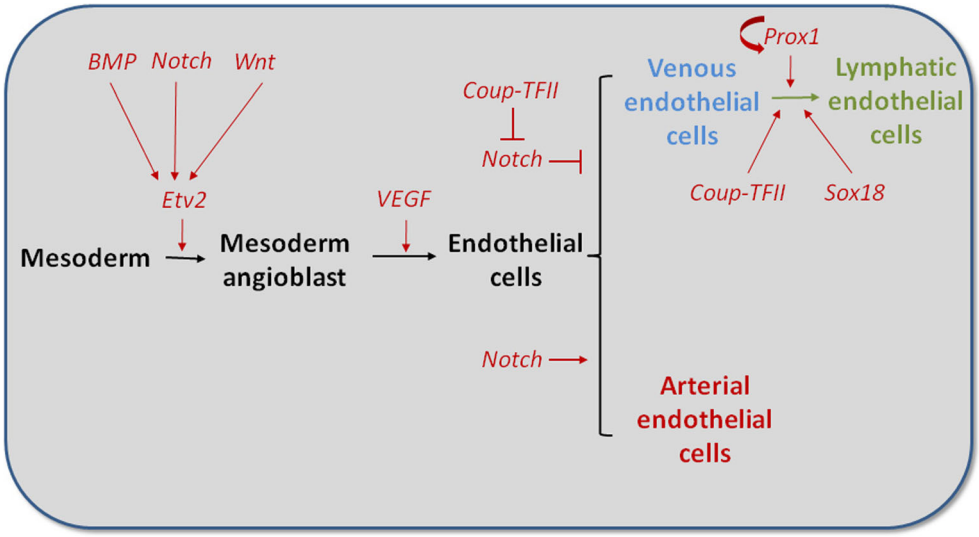

Fig. 1 Lymphatic endotelial cell lineage. Schematic diagram showing some of the major molecular regulators of endothelial cell development and differentiation

At a later stage of development, another important vascular system is originated, the lymphatic system. Venous endothelial cells from the embryonic cardinal vein differentiate into lymphatic endothelial cells (LECs) at stage E9.5 of mice embryogenesis [9, 10]. In zebrafish, the venous origin of LECs seems to be evolutionarily conserved [11]. Other studies in mice show the existence of other cellular precursors in different organs contributing to the lymphatic vasculature in a smaller percentage, such as mesenchymal precursors [12, 13] or hemogenic endothelium [14•, 15, 16].

In the cardinal vein, a specific subpopulation of ECs starts expressing the transcription factor Prox1, becoming progenitors of the entire posterior lymphatic line [17, 18]. The reason why this subpopulation expresses Prox1 is unknown. However, it is known that other transcription factors, such as COUP-TFII and Sox18, are responsible for activating Prox1 in progenitor cells (Fig. 1) [17, 19]. ERK activation is also essential for the development of the lymphatic program since it is involved in the activation of Sox18 and regulates the expression of other key proteins such as vascular endothelial growth factor receptor-3 (VEGFR3) and lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) [20]. Later, progenitor LECs start to proliferate and migrate, guided by VEGF3 and its ligand VEGF-C, giving rise to the first lymphatic sacs [21•]. From them, the whole peripheral lymphatic system sprouts surrounding tissues and organs, where the lymphatic capillaries are established [22]. Then, they begin to express classic markers of the lymphatic lineage necessary for the formation of mature lymphatic network, such as podoplanin, ephrin B2, neurofilin-2, and FoxC2 [23]. The formation of a system composed of internal valves, capillaries and collecting lymphatic vessels prevents lymph recoil. In this context, both VEGF-C and Prox1 continuous signaling are essential to successfully fulfill these processes [21•, 24]. COUP-TFII and Prox 1 interact and regulate VEGFR3 expression. At the same time, VEGFR3 acts as a direct target of Prox1 in vivo, establishing a feedback between VEGFR3 and Prox 1 to maintain the lymphatic phenotype of LECs [25].

Recent discoveries have described further key molecules participating in this process. Studies in knockout mice show an important function of adrenomedullin—known for its potent vasodilator action—in lymphatic development [26]. The activation of Notch1 also plays a role in lymphatic differentiation [27] as well as the expression of some miRNAs, such as, miR-31 [28], miR-181a [29], and miR-466 [30], which are involved in post-transcriptional regulation of Prox1 or FoxC2.

Despite the venous origin of the lymphatic system, the later remains independent from the cardiovascular system. Both are placed very close in tissues, side by side, but with a functional separation to maintain proper angiogenic and lymphangiogenic activities. Despite the few described mechanisms that prevent connection between blood vessels and lymphatics, little is known about this process. For instance, progenitor ECs express SYK protein tyrosine kinase and its substrate SLP-76. This pathway helps to maintain lymphatic vessels integrity and prevents unions between both vasculatures [31, 32]. Podoplanin and CLEC-2 (receptor of platelets) are also involved in this mechanism. Podoplanin activates CLEC-2 and causes platelet aggregation, what leads to activation of SYK and SLP-76 [33, 34]. Another molecule that participates in the proper separation of both vasculatures is an adipose factor, Fiaf. Studies in Fiaf deficient mice showed a reduced expression of Prox1 and found a few blood vessels filled with blood at lymph after birth [35]. Finally, numerous glycoproteins regulate this process of separation between blood and lymphatic vasculatures [36, 37].

Lymphatic Circulation in the Liver

The liver is one of the major lymph-producing organs in the body. Between 25 and 50% of lymph flowing through the

thoracic duct originates in the liver [38••]. Hepatic lymph vessels are either located in the sub-peritoneal fibrous capsule of the liver, Glisson's capsule, or internally in the connective tissue which surrounds branches and portal triad hepatic veins. Most of the lymph originates in the perisinusoidal space of Disse and drains into inner lymphatic vessels in the surrounding area of interlobular portal triads [39]. Both, the superficial and the inner lymphatic vessels of the liver converge towards the hepatic hilum and drain into the lymph nodes arranged along hepatic vessels and hepatic ducts in the lesser omentum.

Lymph Production in Cirrhosis

In liver diseases, the lymphatic system is significantly deteriorated. Lymph flow is greatly increased in cirrhosis, up to 30fold in cirrhotic patients with concomitant increases in the formation of new lymphatic vessels, known as lymphangiogenesis [40•]. Studies of Dumont et al. in cirrhotic patients showed an excessive lymph production during hepatic cirrhosis. Such lymph contains many blood elements due to a higher permeability of hepatic capillaries in this situation [41]. The overproduction of lymph comes primarily from the liver. It is known that lymph produced in the liver has a high concentration of proteins, unlike lymph produced in other organs. In cirrhotic patients and experimental models of hepatic obstruction in dogs, Witte et al. saw that lymph, which increased the flow in the thoracic duct, was characterized by a high protein concentration, indicating that it was originated in the liver [42].

In addition, the number of lymphatic vessels increases in fibrotic and cirrhotic livers. Studies of Corpechot C. et al. and Tugues S. et al. observed increased levels of lymphangiogenesis inducers, such as VEGF-D and VEGF-C, during fibrosis. VEGF-D was positively correlated with progression of liver fibrosis [43, 44]. The increased lymphangiogenesis and vessel enlargement coincides with disease severity [45, 46]. In this context, it has recently been shown that sympathetic nerves are a key regulator of hepatic lymphangiogenesis by secreting VEGF-C in rats with portal hypertension. This link between sympathetic nervous system activation and liver lymphangiogenesis opens a new direction on liver and vascular research [47•].

Size and Density of Lymphatic Vessels in Cirrhosis

In cirrhosis, it has described an increase not only in the number of lymphatic vessels but also in their size, in some cases tripling its area. This phenomenon may be due to increased lymph production caused by cirrhosis. Dumont et al. first reported such effect in cirrhotic patients with abnormal lymphatic vasculature. They observed an increased diameter and lymph flow in the thoracic duct [48]. In the same way, Sadek et al. demonstrated by computed tomography and angiography techniques that lymph vessels in the liver of fibrotic and cirrhotic patients were dilated [49]. In parallel, other teams analyzed at a macroscopic level the superficial hepatic lymphatic vessels by laparoscopy and they reported a dilation of these vessels during hepatic diseases [50]. This expansion was also observed by electron microscopy [51].

Subsequently, in a study of in vivo fluorescence microscopy in rats exposed to CCl_4, Vollmar et al. showed a progressive increase in the number of functional hepatic lymphatic vessels during the first weeks of exposure to CCl_4, peaking at week 12 of treatment. In parallel with the increased density in lymphatic vessels, they observed a progressive increase in vessel area correlating with the stage of disease, stabilizing after 8 weeks of exposure to CCl_4. By immunohistochemistry, they also located new lymphatic vessels aside of portal tracts that were located mainly around the fibrous septa [52]. Consistent with the preclinical studies, this phenomenon was also observed in patients. In histological samples of patients, Yamauchi et al. showed that the area of intrahepatic lymphatic vessels remained stable during early stages of the disease, but it increased significantly when it reached and advanced cirrhosis. In this study, they measured vessel size by visible microscopy and observed an increase in number of lymphatic vessels which correlated with the expansion degree of portal tracts [53]. In addition, Yokomori et al. measured the density of lymphatic vessels in patient specimens by immunohistochemistry. These investigators showed that lymphatic density increased with disease progression, peaking in the most advanced stages of cirrhosis. Moreover, they showed by electron microscopy larger and more visible superficial lymphatic vessels in cirrhotic livers compared to healthy controls [54].

Dysfunction of Lymphatic System in Cirrhosis

There are many evidences of a lymphatic system dysfunction in cirrhosis. For instance, Henriksen JH et al. measured lymphatic conductivity in patients with cirrhosis and in pigs with different hemodynamic alterations of the hepatosplanchnic system. In cirrhotic patients with small amount of ascites, conductance in the thoracic tract was tripled, while in patients with larger and established ascites these values were normalized. Moreover, conductance in lymphatic vessels of cirrhotic patients with ascites was ten times lower compared with that in the thoracic duct. They obtained similar results in animal models of pigs [55]. These complications result in an insufficient lymphatic drainage upon cirrhosis decompensation and ascites accumulation. Ribera et al. evaluated the functionality of the splanchnic and peripheral lymphatic system in an experimental model of rats exposed to CCl_4, by fluorescent lymphangiography. In cirrhotic rats, they found a significant decrease of fluorescence-labeled lymphatics in both,

peripheral and splanchnic areas, demonstrating a dysfunction in lymphatic drainage (Fig. 2) [56].

Role of eNOS in the Dysfunction of Lymphatics in Cirrhosis

Lymphatic capillaries are formed by a thin layer of lymphatic endothelial cells without fenestrations and basement membrane. Unlike the collector lymphatics that lead the lymph through the system, capillaries do not have any coating of SMCs or pericytes. In the absence of a propellant muscular organ, such as the heart in the cardiovascular system, the lymphatic transport is promoted by extrinsic and intrinsic forces. Intrinsic forces come from the contractile capacity of the SMCs layer that surrounds collecting lymphatic vessels and, therefore, these forces have been proposed as the main driver for lymphatic circulation [57]. Regarding the molecular mechanisms governing this contraction, there are evidences

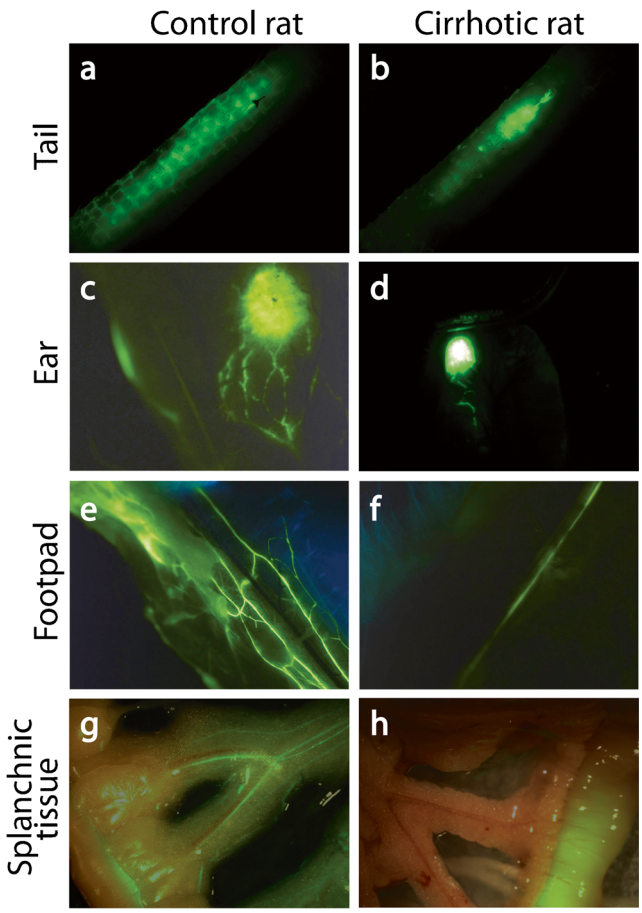

Fig. 2 Impaired lymphatic function in cirrhotic rats. The lymphatic function in control or cirrhotic rats was assessed by fluorescent lymphangiography after injection of 2000 kDa FITC-dextran (panes a to f) and after oral gavage with BODIPY FL C16 day (panels g and h). The representative pictures show a significant decrease in lymphatic functionality associated with the cirrhotic condition

suggesting that lymphatic endothelial cells regulate the contractile ability of SMCs through the production of nitric oxide [58]. In the study of Ribera et al., the expression of eNOS and NO production by lymphatic endothelial cells isolated from control rats were compared with the cirrhotic condition. These investigators found that primary lymphatic endothelial cells isolated from the mesentery of cirrhotic rats overexpressed eNOS compared with cells from healthy animals. Next, they investigated the relevance of this mechanism by blocking the activity of NOS on lymphatics with L-NMMA. This study showed, through lymphangiography, that cirrhotic animals presented a significant improvement in lymphatic uptake after one week of treatment with L-NMMA, both in the peripheral and splanchnic areas. These results indicated that overproduction of NO was involved in the proper functionality of the lymphatic system in cirrhosis. This study established a new mechanism that could explain why inhibition of eNOS activity improved the lymphatic functionality. By immunohistochemistry, it was found that this increase in NO production caused a chronic lymphatic remodeling characterized by the reduction of lymphatic vessels coated with SMCs. In healthy animals, 50% of the lymphatics were covered by SMCs and, therefore, potential responders to vasoconstrictors. In contrast, cirrhotic rats presented only 2.7% of lymphatics coated with SMCs, which represents a significant loss of contractile capacity of collecting lymphatics. This study also demonstrated the pathological role that NO overproduction had over the pericyte population, since NO inhibited the proliferation of SMCs in isolated primary rat SMCs. This interaction between NO and SMCs was investigated by measuring the proliferation of these cells in the presence of NO, quantifying the amount of BrdU captured by flow cytometry. The results of these experiments demonstrated that NO had an antiproliferative effect over this cell type. In this context, the inhibition of eNOS activity with the treatment of L-NMMA also recovered partially the coating of lymphatic vessels with SMCs [56].

Contribution of the Lymphatic System to the Formation and Accumulation of Ascites One of the mechanisms of ascites formation in cirrhosis is the excessive formation of interstitial edema that exceeds the capacity of the lymphatic system for draining splanchnic and hepatic lymph back to the blood circulation. In this context, the fluid formed in the interstitial spaces escapes and gets into the peritoneal cavity where it accumulates. This increased liver lymph flow contributes to the accumulation of ascites [41]. The first theory linking the lymphatic system to the accumulation of ascites was suggested by Witte et al. in 1980, when they formulated the theory known as "lymph imbalance." This theory proposed that there is an imbalance between the volume of lymph produced in cirrhosis and the volume of lymph returned into blood circulation [59]. This imbalance is responsible for the wrong distribution of the extracellular fluids that stimulates renal

retention of salt and water and, as a consequence, the accumulation of ascites. Compared with the lymphatic flow in healthy subjects (1 L/day), previous publications demonstrated a significant increase in lymph flow in the thoracic duct of cirrhotic patients with cirrhosis (8–9 L/day). Thus, the system is capable of draining a moderate amount of lymph and returns it back to systemic circulation, preventing accumulation of fluid in the abdominal cavity. But as cirrhosis progresses, there is an imbalance between the production of lymph and the capacity of the lymphatic system to return it back into systemic circulation, so the lymphatic system cannot compensate the increase of lymph resulting in the accumulation of ascites in the peritoneal cavity [60]. The onset of this impaired lymphatic drainage in patients with dysfunctional livers has previously been explained by the passive saturation of lymphatics with excessive lymph formation and the anatomical limitations of the connectivity between the lymphatic and cardiovascular systems. However, this conclusion has been challenged by studies suggesting the participation of other mechanisms that impair lymphatic conductivity in decompensated cirrhotic patients compared with compensated cirrhotic patients [55]. Results from our laboratory also support the contribution of active mechanisms in the pathophysiology of lymphatic dysfunction. For instance, Ribera et al. demonstrated that the inhibition of the lymphatic nitric oxide synthase improved lymphatic functionality and reduced ascites volume in the peritoneal cavity of cirrhotic rats. This reduction of ascites was a consequence of an increased reabsorption of fluid from the peritoneal cavity into the cardiovascular system [56].

Cirrhosis can also drive to the formation of another type of ascites, in this case due to disruption or obstruction of the lymphatic circulation. In this context, the intestinal lymph (chyle) is accumulated in the peritoneal cavity and leads to the appearance of chylous ascites. In fact, 60% of cases of chylous ascites in the Western world are due to liver cirrhosis. Chylous ascites occurs in 0.5–1% of patients with cirrhosis and ascites; it also can appear as a symptom or a late consequence of hepatocellular carcinoma. Pathophysiologically, this kind of ascites appears when the lymph vessels breakdown because of the dilatation originated by the increased pressure in the portal vein with an excessive lymph flow [61–63]. All together, these observations suggest that lymphatic impairment in liver disease may have diverse causes and, therefore, a deep characterization of the lymphatic disorder is clinically relevant.

Conclusions

Lymphatic research in hepatology has been scarce for a variety of reasons including the difficulty of locating and cannulating lymphatic vessels, which can be significantly invasive for clinical research and nearly impossible in basic science. However, our understanding of the pathogenesis of lymphatic dysfunction in liver disease has advanced significantly in the last decade due to the development of new methodology to interrogate lymphatic function and the availability of specific markers for lymphatic endothelial cells. Thanks to these improvements, scientists have identified novel pathological mechanisms and have developed new research lines addressed to correct structural changes and function affecting lymphatic vessels in the context of liver disease. Despite the knowledge gained, we still have to face many unresolved questions concerning the pathological role played by the lymphatic system in the pathogenesis of chronic liver disease and the design of therapeutic targeting. To this end, more basic and clinical research is needed. It is expected that the accumulation of knowledge, together with further efforts to answer unresolved questions, will eventually lead to the development of safer and effective therapies to treat the described lymphatic dysfunction in this clinical condition.

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Compliance with Ethical Standards

Conflict of Interest Jordi Ribera, Bernat Córdoba-Jover, Irene Portolés and Manuel Morales-Ruiz each declare no potential conflicts of interest.

Human and Animal Rights and Informed Consent All reported studies/experiments with human or animal subjects performed by the authors have been previously published and complied with all applicable ethical standards (including the Helsinki declaration and its amendments, institutional/national research committee standards, and international/national/institutional guidelines).

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- •• Of major importance

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