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Review article

Lymphatic and blood systems: Identical or fraternal twins?

Florent Morfoisse, Agnès Noel*

Laboratory of Tumor and Development Biology, GIGA (GIGA-Cancer), Liege University, B23, Avenue Hippocrate 13, 4000, Liege, Belgium

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ABSTRACT

Blood and lymphatic systems work in close collaboration to ensure their respective physiological functions. The lymphatic vessel network is being extensively studied, but has been overlooked as compared to the blood vasculature mainly due to the problematic discrimination of lymphatic vessels from the blood ones. This issue has been fortunately resolved in the past decade leading to the emergence of a huge amount of data in lymphatic biology revealing many shared features with the blood vasculature. However, this likeliness between the two vascular systems may lead to a simplistic view of lymphatics and a direct transcription of what is known for the blood system to the lymphatic one, thereby neglecting the lymphatic specificities. In this context, this review aims to clarify the main differences between the two vascular systems focusing on recently discovered lymphatic features.

1. Introduction

In addition to blood vessels, humans and most of the mammals exhibit a second vascular system: the lymphatic vasculature. This vascular system is known since the seventeenth century thanks to the work of Gaspare Aselli who identified them in the intestine of well-fed dogs and introduced them as “milky veins” or “lacteals” (Suy et al., 2016). Nevertheless, modern researches on lymphatics have been hampered for a long time by the absence of reliable methods to discriminate lymphatic and blood vessels. The identification of three key specific lymphatic markers in the early 2000’s has solved this issue. First, Wigle et al identified the Prospero homeobox protein 1 (Prox1) as the main lymphatic transcription factor controlling the acquisition and upkeep of the lymphatic fate during development (Wigle and Oliver, 1999). Forced expression of Prox1 is sufficient to trigger lymphatic reprogramming even in adult blood endothelial cells (BECs), whereas its deletion induces a loss of lymphatic identity and a phenotypic switch from lymphatic endothelial cells (LECs) to BECs. Therefore, Prox1 is viewed as a key gatekeeper of the lymphatic fate (Hong et al., 2002; Johnson et al., 2008). In addition to Prox1, two specific extracellular receptors were identified at the same time: the lymphatic vascular endothelial-cell hyaluronan receptor-1 (LYVE-1) (Prevo et al., 2001; Banerji et al., 1999), an homolog of the CD44 receptor and podoplanin, a transmembrane glycoprotein (Breiteneder-Geleff et al., 1999). These lymphatic markers have allowed the investigation of specific features of lymphatic vessels. The lymphatic system turned out rapidly to be worth studying as its absence is incompatible with life. Indeed, it ensures three major physiological processes: (i) it contributes to the uptake of dietary lipids through the intestinal lymphatic vasculature and the chylomicron transport (Randolph and Miller, 2014; Dixon, 2010; Bernier-Latmani and Petrova, 2017), (ii) it participates to immune surveillance, inflammation and graft rejection (Patel and Dana, 2009; Randolph et al., 2017; Kim and Song, 2017) thanks to its capacity to coordinate the trafficking of antigen and immune cells and finally (iii) it regulates tissue fluid homeostasis (Olszewski, 2003; Tammela and Alitalo, 2010). Similarly to the blood vasculature, the lymphatic system is composed of capillaries and larger vessels called collecting vessels that differ in their structure and functions. Nowadays, the lymphatic vasculature is extensively studied and innovative data keep emerging rapidly. Notably, a lymphatic network in the meningeal space has been recently described

Abbreviation: ADAMTS3, a disintegrin and metalloprotease with thrombospondin motifs-3; BEC, blood endothelial cell; CAD, coronary artery disease; CCBE1, collagen- and-calcium binding EGF domains 1; CNS, central Nervous System; CPT1A, carnitine palmitoyltransferase 1A; ECM, extracellular matrix; Flt4, fms-like tyrosine kinase 4; GATA2, gATA-binding protein 2; GLUT1, glucose Transporter 1; JNK 1/2, c-JUN N-terminal kinase-1/2; LEC, lymphatic endothelial cell; LN, Lymph node; LYVE-1, lymphatic vascular endothelial-cell hyaluronan receptor-1; MMP-2, matrix metalloproteinase-2; MT1-MMP, membrane type 1-matrix metalloproteinase; NRP1/NRP2, semaphorin receptor neuropilin 1/2; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3; PKC, phospholipase-Cγ (PLCγ)/protein kinase C; Prox1, prospero homeobox protein 1; VEGF-A, vascular Endothelial Growth Factor-A; VEGF-C, vascular Endothelial Growth Factor-C; VEGF-D, vascular Endothelial Growth Factor-D; VEGFR, vascular Endothelial Growth Factor Receptor; VPF, vascular Permeability Factor; uPARAP, urokinase plasminogen activator receptor-associated protein

* Corresponding author.
E-mail address: agnes.noel@uliege.be (A. Noel).

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(Antila et al., 2017; Aspelund et al., 2015) opening new options for the treatment of neuronal pathologies such as Alzheimer disease (Da Mesquita et al., 2018). In addition, the plasticity of the lymphatic network has been highlighted with the identification of specific, highly specialized lymphatic profiles depending on their location: lacteals in the intestine (Bernier-Latmani et al., 2015) and ascending vasa-recta in the kidney for instance (Kenig-Kozlovsky et al., 2018). A better understanding of the lymphatic system revealed that it shares many similarities with the blood vasculature in terms of development, growth factor sensitivity and signalling pathways implicated during cellular proliferation, migration and formation of new vessels. Nevertheless it is now well accepted that data obtained on blood vessels are not directly applicable to lymphatics. Since the lymphatic system is now more and more recognized as worth studying per se, the authors consider that it is important to clarify the specificities of the lymphatic system especially during pathological conditions. The aims of this review is thus to provide a summary of the main differences between blood and lymphatic systems to the readers with a particular emphasis on lymphatics specificities in term of structure, signalling pathways and molecular regulators.

2. Specificities of lymphatic vessels at the morphological level

Blood and lymphatic systems share many features: during the development, precursors of lymphatic cells emerge among BECs in the cardinal veins and migrate from it to form the initial lymphatics (Srinivasan and Oliver, 2011; Yang et al., 2012; Hagerling et al., 2013), while in adults both vascular networks share the same hierarchical organization, small and numerous capillaries merging into some larger vessels forming the so-called “vessel tree structure” and are most of the time in close proximity. In addition, the two systems communicate in various body areas such as in lymph nodes and in the subclavian vein where the lymph goes back to the blood circulation. However, blood and lymphatic systems display very distinct functions, the former being a provider of oxygen and nutrients, and the latter being implicated in immune surveillance, fat transport and interstitial fluid drainage (Tammela and Alitalo, 2010). Each vascular network has thus developed specialized structural adaptions in order to ensure their respective physiological roles.

2.1. Lymphatic capillaries

The interstitial fluid, which is the precursor of the lymph, is formed as an ultrafiltrate of capillary microcirculation. According to the Starling model, hydrostatic and osmotic pressure gradients drive the fluid filtering from the arterial part of the blood circulation into the interstitial space, while it is later reabsorbed into the venous part, lymphatic vessels removing the remainder (Starling, 1896; Michel, 1997). This concept has been revised as more precise measurements revealed that fluid extravasation occurs at both the arterial and the venous parts of the blood circulation into the interstitial space (Levick and Michel, 2010; Levick, 1991). Fluids are then absorbed by lymphatic capillaries, also called initial lymphatics, which are responsible for the fluid drainage properties of the lymphatic vasculature. They are specifically designed to be an easy entry point for fluids and macromolecules. They are thin-walled and blind-ended vessels composed of a single layer of LECs, which are not continuously covered by pericytes or smooth muscle cells like their blood counterpart (Fig. 1A, B). Furthermore, contrary to continuous junctions between endothelial blood cells, lymphatic capillaries display very specific intercellular discontinuous junctions called “button-like” junctions characterized by parallel linear segments of Vascular Endothelial cadherin (VE cadherin) displaying a typical oak-leaf shape (Fig. 1C). The interjunctional gaps between these buttons allow fluids and macromolecules to passively enter the vessel (Baluk et al., 2007). It has been recently reported that Angiopoietin 2 is one of the protein critically implicated in their formation (Zheng et al., 2014). Button junctions are acquired shortly after birth and are strictly restricted to initial lymphatics. In contrast, collecting lymphatic vessels display only continuous zipper-like junctions. Interestingly, the type of LEC junctions in a lymphatic capillary is plastic: a growing lymphatic sprout first exhibits zipper-like junctions and then acquire button-like ones, while the reverse switch from button to zipper junctions can be induced at least by inflammation (Yao et al., 2012). Changes in fluid balance also directly affect LEC junctions: an over-hydration for example, has been demonstrated to decrease VE-cadherin and PECAM expression (Miteva et al., 2010). Another unique feature of initial lymphatic is the incomplete or even absence of basal membrane (Schulte-Merker et al., 2011). While BECs lie on basement membrane components (mainly laminin and type IV collagen), LECs are intimately associated with the interstitial matrix mainly composed of type I collagen (Paupert et al., 2011) (see part 3.B). LECs are connected to the surrounding tissue by anchoring filaments, which attach to interstitial type I collagen fibers and are mainly composed of emilin-1 and fibrillin (Danussi et al., 2008; Maby-El Hajjami and Petrova, 2008). These filaments actively participate to the drainage of the excess of extracellular fluids: an increased interstitial pressure could stretch the connective tissue fibers and anchoring filaments, pull adjacent endothelial cells apart and increase the diameter of lymphatic vessels (Ji, 2006). Consequently, the disruption of these filaments observed in emilin-1$^{-/-}$ mice results in defects of lymphatic vasculature inducing a strong inhibition of lymphatic fluid drainage, lymph leakage and lymphedema development (Danussi et al., 2008).

2.2. Collecting lymphatic vessels

Collecting lymphatic vessels are responsible for the transport of the lymph and cells back to the blood circulation through the subclavian veins (Tammela and Alitalo, 2010). They are thus less implicated in fluid uptake, which is reflected by their structure. Indeed, LECs of collecting vessels exhibit only zipper-like intercellular junctions and these vessels are fully covered by pericytes and smooth muscle cells (Baluk et al., 2007). Both lymphatic and blood vessels have to convey liquid throughout the body. While the heart ensures the propulsion of the blood through the blood vasculature, the lymphatic system lacks a heart-like central pump to move lymph inside the vessels. Indeed, the pulsatile function of the lymphatic system relies on the collecting vessels themselves. These vessels are divided into functional contractile units called lymphangion separated by valves similar to the ones found in veins that prevent lymph backflow (Bazigou et al., 2009). These valves are key elements for vessel functionality and as such, have been extensively studied (Sabine and Petrova, 2014; Bazigou et al., 2014; Vittet, 2014; Pujol et al., 2017; Tatin et al., 2013; Levet et al., 2013). The synchronized contractions of lymphangions throughout the vessel resulting from the contraction of both their muscle cells and the surrounding skeletal muscles allow the transport of the lymph inside the lymphatic network. Fluid is returned to the blood circulation both in lymph nodes and via larger lymphatic trunks such as the thoracic duct (Breslin, 2014). This “lymphatic pump” can ben dynamically regulated by various signals (Scallan et al., 2016; Padera et al., 2016; van Helden, 2014).

2.3. Tissue heterogeneity in lymphatic vasculature

Blood and lymphatic networks display an important heterogeneity in terms of origins and gene expression reflecting several distinct organ-specific specialized functions (Aird, 2012; Marcu et al., 2018; Petrova and Koh, 2018). Recent studies based on lineage tracing have indeed highlighted multiple unexpected origins for lymphatic vasculature of different tissues/organs (Petrova and Koh, 2018). It includes, at least, (i) Tie2-lineage^{+} venous-derived LECs progenitors (Martinez-Corral et al., 2015) for skin, cervical and thoracic lymphatic vessels; (ii) Tie2-lineage– nonvenous LECs progenitors for most lumbar and some cardiac

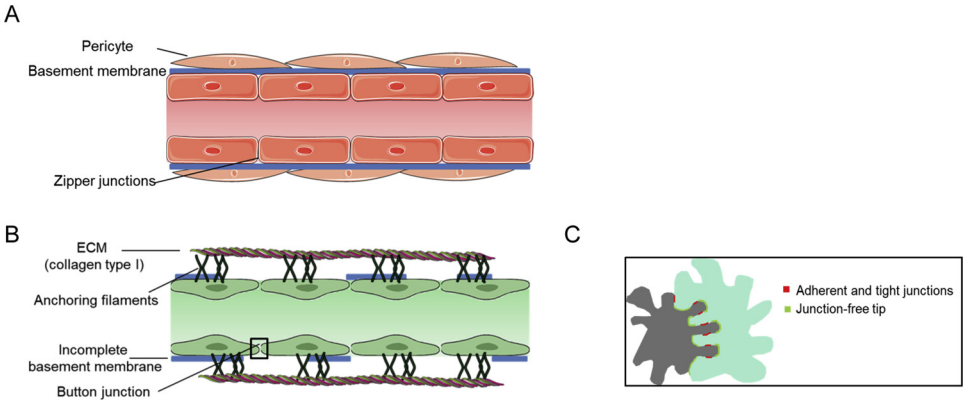

Fig. 1. Structural properties of blood and lymphatic capillaries.
A: Schematic representation of a blood capillary. Blood endothelial cells display only zipper junctions. Blood capillaries exhibit a continuous basement membrane and are covered by pericytes. B: Schematic representation of a lymphatic capillary. Lymphatic cells are connected through distinctive, discontinuous button-like junctions. Lymphatic vessels interact with the ECM through anchoring filament and are poorly covered by the basement membrane. C: Detailed view of button-like junctions showing that LECs exhibit a specific oak leaf – shaped profile with complementary shapes and overlapping edges. Adherent junctions and tight junctions at the sides of flaps (in red) allow fluid entry to the junction-free region at the tip (in green) without repetitive disruption and reformation of junctions.

lymphatic vessels (Tatin et al., 2017; Klotz et al., 2015); (iii) nestin^{+} precursors in LN vessels (Koning et al., 2016). Further studies are required to determine the respective roles of each LEC subpopulation originating from several cellular sources and forming lymphatic vessels of a tissue/organ.

All of the organs were thought to possess both specialized blood and lymphatic vessels excluding the central nervous system (CNS). Until recently, the CNS was considered to lack lymphatic vessels raising the question about how cerebral interstitial fluid, containing metabolites and waste products from the brain, was cleared (Hladky and Barrand, 2014; Nedergaard, 2013). The cerebrospinal fluid of the CNS was initially identified as a key player of brain drainage (Hladky and Barrand, 2016). This fluid forms in the choroid plexi, flows through the cerebral ventricles and drains to the subarachnoid space where it is reabsorbed into the bloodstream (Damkier et al., 2013; Sykova and Nicholson, 2008; Praetorius, 2007). Interstitial solutes have been thought to be cleared to the cerebrospinal fluid by passive diffusion from the interstitial fluid (Abbott, 2004). However, studies based on injections of tracer molecules into rat brains revealed that the injected compounds did not diffuse homogenously, but rather drained via preferential pathways, specifically the perivascular spaces of cerebral blood vessels (Cserr et al., 1977; Cserr and Ostrach, 1974). More precisely, cerebrospinal fluid enters the brain specifically within the perivascular spaces of cerebral arteries, whereas interstitial fluid drains from brain exclusively alongside the perivascular spaces of large calibre veins (Iliff et al., 2012). In the same studies, the authors demonstrated that astrocytes are required to form these perivascular spaces and to ensure their function. Therefore, the authors named this pathway, the glial-associated lymphatic system, or glymphatic system.

A couple years ago, two independent studies identified the presence of a lymphatic network in the dura matter, the external layer of the meningeal linings, in mice (Aspelund et al., 2015; Louveau et al., 2015). Interestingly, these meningeal lymphatic vessels develop postnatally, appearing first at P16 around the foramina in the basal part of the skull (Antila et al., 2017). They sprout along the blood vessels as their development heavily relies on the Vascular Endothelial Growth Factor-C (VEGF-C)/Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3) pathway, VEGF-C being produced by smooth muscle cells surrounding the blood vessels (Antila et al., 2017). Meningeal lymphatics have been identified to be responsible for the drainage of cerebrospinal fluid from the subarachnoid space to the lymphatic circulation through the deep cervical lymph nodes (Aspelund et al., 2015; Louveau et al., 2015). In line with this physiological function, these vessels display several features of lymphatic capillaries, such as a lack of smooth muscle coverage and lymphatic valve associated with a discontinuous basement membrane and the presence of anchoring filaments associated to the ECM (Louveau et al., 2015; Koina et al., 2015). Taken together, these new data reveal a two-steps process allowing the fluid drainage in the brain: first, cerebrospinal fluid produced in the subarachnoid space, flows through the brain and allows solute exchange with the brain interstitial fluid via the glymphatic system, then once returned to the subarachnoid space, it reaches the lymphatic circulation through the dural lymphatic system and the deep cervical lymph nodes ensuring its clearance.

It is worth noting that a novel type of dispersed nonlumenized LEC-like cells has been identified in the brain of zebrafish by three different groups in 2017 (Venero Galanternik et al., 2017; Bower et al., 2017a; van Lessen et al., 2017). Those cells surrounding meningeal blood vessels are called “muLECs” or “mannose receptor-1 + perivascular cells”. Further studies are needed to determine their roles in zebrafish and existence in mammals

These new data has raised the question of the implication of lymphatic drainage in neurodegenerative disorders and in particular in Alzheimer’s disease. Ageing is the principal risk factor for many neurological disorders, including Alzheimer’s disease (Erkkinen et al., 2018; Brookmeyer et al., 2018) and it has been demonstrated that impaired brain perfusion by cerebrospinal fluid and cognitive functions in old mice was accompanied by a decrease in meningeal lymphatic vessel diameter and coverage, as well as decreased drainage of cerebrospinal fluid macromolecules into cervical LNs (Da Mesquita et al., 2018). Lymphatic-mediated drainage of the cerebrospinal fluid seems to be directly related to cognitive function as damaging the meningeal lymphatics in young mice resulted in learning and memory deficits. On the opposite, inducing lymphangiogenesis through VEGF-C administration in old animals improved their cognitive functions (Da Mesquita et al., 2018). In-depth studies must still be performed to evaluate the implication of meningeal lymphatics in patients suffering from neurodegenerative disease. Nevertheless these data suggest that improving the lymphatic draining function might represent a therapeutic strategy at least in Alzheimer’s disease. Another interesting aspect in the field is how this cerebral lymphatic vasculature could be used to facilitate drug delivery throughout the CNS. Indeed, the blood-brain barrier restricts macromolecules entering the CNS. By taking advantage of the brain lymphatic drainage pathways (glymphatic system and dural lymphatic network), therapeutic agents could be more efficiently been transported throughout the brain (Sun et al., 2018). Currently two main administration routes are extensively studied. First, ependymal cells, a layer of glial cells have been transfected with viral vectors allowing a glymphatic system-mediated drug delivery into the cerebrospinal fluid (Achariyar et al., 2016; Yamazaki et al., 2014). Alternatively, intranasal drug administration will use the lymphatic system of the nasal mucosa to gain access to the cerebrospinal fluid bypassing the blood-brain barrier (Kim et al., 2014; Miyake and Bleier, 2015).

3. Specificities of LECs at the cellular levels

3.1. Similar appearance, opposite metabolism

At the cellular level, BECs and LECs share many similarities when forming new vessels. Both of them require an external stimulation (by growth factors or cytokines) to form an initial sprout where cells acquire two different profiles: the tip cells produce numerous filopodia to sense the gradient of growth factor and guide the sprouting process toward it, followed by stalk cells proliferating to form the new vessel (Gerhardt et al., 2003). The balance between the two phenotypes is mostly controlled by the lateral inhibition exerted by the tip cell on neighbouring cells through the Notch pathway (Phng and Gerhardt, 2009). Currently, the role of Notch in lymphatic sprouting is not as clear as in angiogenesis, although it seems playing a similar role at least when lymphangiogenesis is triggered by VEGF-A (Zheng et al., 2011). One might also consider that both systems share the same metabolism. However the two types of endothelial cells display opposite metabolism. BECs are highly glycolytic (De Bock et al., 2013; Parra-Bonilla et al., 2010) and rely mainly on a glycolytic metabolism driven by a Glucose Transporter 1 (GLUT1)-dependent glucose uptake despite being also able to switch to oxidative metabolism if needed (Dranka et al., 2010; Mann et al., 2003). Given the far better efficiency of the mitochondrial respiration than glycolysis in term of ATP production, it may seems counterintuitive to rely on it especially for BECs that have free access to oxygen to extensively use oxidative phosphorylation. However, a glycolytic metabolism is well adapted to the physiological functions of BECs: being anaerobic, they can sprout into an avascular, hypoxic environment where interstitial glucose levels are not rate-limiting thus ensuring the furniture of oxygen to the tissues lacking it (58, 188). Since it is not consumed by oxidative phosphorylation in BECs, the oxygen amount for transfer to surrounding cells and tissues is preserved. A high rate of glycolysis will also generate a high amount of lactate that acts as a pro-angiogenic molecule (Hunt et al., 2007; Ruan and Kazlauskas, 2013). This has been particularly well observed in cancers where lactate produced by tumor cells is extensively used by endothelial cells (Polet and Feron, 2013). Furthermore, angiogenic growth factors such as VEGF-A activate several inducers of glycolysis such as GLUT1 (van Beijnum et al., 2006; Yeh et al., 2008). Aside from serving as an energy source, the glycolytic metabolism of BECs is also required for the vessel organization and the acquisition of the tip/stalk cell profile. VEGFs stimulation upregulates the expression of glycolysis regulator 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), an activator of phosphofructokinase 1 in the tip cell and thus increased the glycolytic flux. At the opposite, the activation of the pro-stalk signalling Notch receptor via Dll4 reduces PFKFB3-driven glycolysis in the surrounding cells (De Bock et al., 2013).

Contrary to BECs, it has been recently reported both, in mice and zebrafish that LECs heavily rely on fatty acid oxidation (FAO) (Wong et al., 2017; Zecchin et al., 2018). Interestingly, more than being required simply for providing energy, FAO is essential to the acquisition and the upkeep of lymphatic phenotype. Indeed, while the silencing of carnitine palmitoyltransferase 1A (CPT1A) did not induce energy distress in LEC, mice genetically deleted for this enzyme exhibit severe lymphatic defects (Wong et al., 2017). In fact, Wong et al demonstrated that the role of FAO in LECs is to produce acetyl-CoA. Accordingly, an acetate supplementation can restore lymphangiogenesis upon FAO inhibition. Acetyl-CoA/CoA ratios are already known to be crucial for histone acetylation by histone acetyltransferase p300 (Lee et al., 2014) but, for the first time, the authors revealed that Prox1 binds p300 and promotes histone acetylation on lymphangiogenic genes. Thus, during BEC to LEC differentiation, Prox1 induces the overexpression of CPT1A to promote FAO increasing acetyl-CoA production. Acetyl-CoA is then used as a substrate for histone acetylation mediated by p300. By interacting with p300, Prox1 also allows chromatin decondensation at lymphangiogenic genes, thereby promoting gene transcription. It is worth noting that this metabolic profile seems to be evolutionary conserved as it has also been observed during the embryonic development in the zebrafish (Klotz et al., 2015). Given the metabolic features of blood and lymphatic systems, targeting their metabolism seems to be a good strategy, particularly if one aims to inhibit or induce one of the vasculature without affecting the other. FAO inhibition has already been successfully used in melanoma to reduce tumoral lymphangiogenesis and metastatic spread (Bastos et al., 2017) thus pointing out the key actor of metabolism as promising therapeutic targets.

3.2. Endothelial cells-extracellular matrix interactions

BECs and LECs cells exhibit several differences in their interactions with extracellular matrix (ECM) (Paupert et al., 2011; Wiig et al., 2010). Due to the incomplete or absent basal membrane in capillaries, LECs are more directly in contact with interstitial matrix through anchoring filaments, which induce specific behaviour depending on which matrix composition there are cultivated on. For example, LECs display higher survival and tubulogenesis on type I collagen without exogenously added growth factors, whereas BECs poorly survived in the same environment (Podgrabinska et al., 2002). Furthermore, in the presence of growth factors and shear stress induced by flow, BECs organize better in a matrix composed of equal amount of collagen and fibrin, whereas LECs prefer a matrix composed only of fibrin (Helm et al., 2007). ECM components or their degradation products, as well as proteins implicated in matrix remodelling such as matrix metalloproteinases (MMPs) are well known to regulate angiogenesis (Kojima et al., 2008; Prats et al., 2013). Less is known in the context of lymphangiogenesis. Interestingly, MMP-2 is involved in lymphangiogenesis in both mice and zebrafish and drives LEC migration in a type 1 collagen matrix by acting as an interstitial collagenase (Detry et al., 2012). At the opposite, membrane type 1-matrix metalloproteinase (MT1-MMP) is able to cleave the lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) on LECs and to inhibit LYVE-1-mediated lymphangiogenic responses. It also restrains the production of VEGF-C in macrophages through repressing the activation of NF-kB signalling (Wong et al., 2016). In addition, matrix stiffness itself could regulate lymphangiogenesis. Indeed, a decrease of matrix stiffness has been associated with an increased expression of the transcription factor GATA-binding protein 2 (GATA2), which is required for the migration of progenitor LECs from the cardinal veins during the development (Frye et al., 2018). GATA2 also controls LEC responsiveness to VEGF-C, the main lymphangiogenic factor (Frye et al., 2018). Most of the effects of ECM components on angiogenesis and lymphangiogenesis are mediated via integrins. Integrins are a family of transmembrane glycoproteins critically implicated in cell–cell and cell–ECM connections composed of 18 large (α) and 8 small (β) subunits generating 24 different heterodimers (Avraamides et al., 2008; Garmy-Susini and Varner, 2008). Integrins act as transmembrane linkers between their ECM ligands and the intracytoplasmic partners allowing signal transduction leading to cell adhesion and migration. One of the most important integrin for the lymphatic system is the α9 integrin subunit. It binds directly VEGF-C and VEGF-D (Vlahakis et al., 2005) and has been reported to control LEC chemotaxis at least in a VEGF-C gradient (Mishima et al., 2007). More precisely, integrin α9β1 is required for the development of lymphatics as mice genetically deleted for this integrin die early due to chylothorax (Huang et al., 2000). Interestingly, a mutation in α9 integrin subunit gene has also been associated with chylothorax development in human foetuses (Ma et al., 2008). Lymphatic defects observed in mice deleted for α9 integrin is due to an abnormal valve formation caused by a defective fibronectin matrix organization (Bazigou et al., 2009). Integrin α9 regulates also lymphangiogenesis in adults in the inflamed cornea (Altiok et al., 2015). In this context, its blockade represents a potential therapeutic strategy to promote transplant survival (Kang et al., 2016). Despite its role in VEGF-A mediated angiogenesis (Staniszewska et al., 2007), mice deleted for integrin α9 subunit do not exhibit any blood vascular defect

thereby suggesting a closer association of this integrin with the lymphatic system. Two other fibronectin binding integrins, α4β1 and α5β1 promote lymphangiogenesis (Garmy-Susini and Varner, 2008; Dietrich et al., 2007; Okazaki et al., 2009). While α4β1 is more implicated in tumoral lymphangiogenesis, promoting metastatic dissemination through the formation of pre-metastatic niches in lymph nodes (Garmy-Susini et al., 2013, 2010), the role of α5β1 has been more described in the context of inflammatory lymphangiogenesis in the cornea and airways (Dietrich et al., 2007; Okazaki et al., 2009). In addition to lymphangiogenesis, both α4β1 and α5β1 are also implicated in tumoral angiogenesis (Garmy-Susini et al., 2005; Boudreau and Varner, 2004; Kim et al., 2000) reflecting that integrins, and in particular the β1 family can also have an equivalent role in the two different vasculatures.

4. Specificities in vascular signalling pathways

4.1. Growth factors

Among many growth factors that could exhibit angiogenic or lymphangiogenic properties, growth of blood and lymphatic vessels is mainly regulated by the VEGF family. This family is composed of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and the placental growth factor (Ferrara, 2004). All family members stimulate proliferation and migration of BECs in vitro and it has been shown that at least VEGF-A,VEGF-C, and VEGF-D act on LECs as well (Yamazaki and Morita, 2006; Shibuya, 2011; Melincovici et al., 2018). However, these factors exhibit also endothelium-specific properties.

4.1.1. VEGF-A

VEGF-A, which was initially called vascular permeability factor (VPF), is a homodimeric glycoprotein with a molecular weight of approximately 45 kDa. At least 9 VEGF isoforms exist as a result of alternative patterns of splicing (Arcondeguy et al., 2013). Three of these, the VEGF isoforms of 121, 165, and 189 amino acids, are preferentially expressed by VEGF-A–producing cells (Tischer et al., 1991). Each of the isoforms contributes to form a VEGF-A gradient essential for proper endothelial cells migration. Considered as the main angiogenic factor, VEGF-A is required for all the steps of blood vessel formation such as: chemotaxis and differentiation of endothelial precursor cells (EPCs), EC proliferation and their assembly into vascular structures (vasculogenesis) or their remodelling (Adams and Alitalo, 2007). Among others angiogenesis stimulators, hypoxia is mainly responsible for the overexpression of VEGF-A (Morfoisse et al., 2015). Under normoxia, prolyl hydroxylase domain protein 2 (PHD2) uses oxygen to hydrolyze HIF1 leading to its degradation (Tervala et al., 2015). Under hypoxic conditions, PHD2 becomes inactive, allowing HIF1 to escape degradation and to bind hypoxia response elements (HREs), a DNA sequence motif present in HIF target genes. HIF1 will thus increase the transcription of VEGF-A (Durre et al., 2018; Parker et al., 2012). Interestingly, hypoxia is able to control VEGF-A expression both at the transcriptional and at the post-transcriptional level. Indeed, VEGF-A mRNA possess an Internal Ribosome Entry Site (IRES), a secondary structure that allows a cap-independent translation, activated under hypoxia (Teran and Nugent, 2015; Hoeben et al., 2004).

VEGF-A displays also some lymphangiogeneic properties (Morfoisse et al., 2015). Notably, VEGF-A overexpression was associated with an increase of tumoral lymphangiogenesis, enlargement of peritumoral lymphatic vessels and lymphatic metastasis (Hirakawa et al., 2005). Moreover, VEGF-A is able to promote corneal lymphangiogenesis indirectly by recruiting macrophages that produce a huge amount of VEGF-C and-D and therefore drive lymphatic growth (Cursiefen et al., 2004). VEGF-A can also directly stimulate LECs leading to a strong and rapid induction of extracellular signal-regulated kinases (ERK) pathway that triggers LECs proliferation and migration (Deng et al., 2015)

4.1.2. VEGF-C

VEGF-C is the lymphatic counterpart of VEGF-A being an essential factor for the development and maintenance of vessels. Mice deficient for VEGF-C develop functional blood vessels but die due to a complete absence of lymphatic vessels and even VEGF-$C^{+/-}$ mice display severe lymphatic hypoplasia (Karkkainen et al., 2004). Normally differentiating in the cardinal vein and emerging from it during the development, LECs in VEGF-C null mice fail to migrate to form the primary lymph sacs leading to complete abrogation of the lymphatic network formation. A strict balance in VEGF-C levels is required to ensure proper lymphatic function: VEGF-C overexpression being directly associated with pathologies caused or at least reinforced by excessive lymphangiogenesis such as metastatic spread (Skobe et al., 2001; Hirakawa et al., 2007), Gorham-Stout syndrome(Hominick et al., 2018) or graft rejections (Mimura et al., 2001; Diamond et al., 2018). A forced expression of VEGF-C is also sufficient to induce experimental models of pathologies like lymphangiectasia (Baluk et al., 2017).

Contrary to VEGF-A, VEGF-C is first produced as full length preform of 58 kDa, which binds only to VEGFR-3. This pre-VEGF-C form needs several cleavages to be fully functional. This processing does not only increase its affinity for VEGFR-3 but allows its binding to VEGFR-2 once in its mature 21 kDa form. VEGF-C processing is mediated either by plasmin (McColl et al., 2003) or by an association of the collagen- andcalcium binding EGF domains 1 (CCBE1) protein and the A disintegrin and metalloprotease with thrombospondin motifs-3 (ADAMTS3) protease (Jeltsch et al., 2014). Mutations in genes coding for these two proteins impaired functions of VEGF-C and lead to severe pathologies linked to a loss of lymphatic function (Alders et al., 2009; Brouillard et al., 2017).

Although not as potent as VEGF-A, VEGF-C also exhibits a pro-angiogenic activity. It regulates the formation of vascular beds both in vitro and in vivo. However, vascular defects induced by VEGF-C deficiency can be easily restored by VEGF-A supplementation confirming the central role of VEGF-A in angiogenesis (Cao et al., 1998; Hamada et al., 2000).

4.1.3. VEGF-D

VEGF-D, also known as c-fos induced growth factor, shares many features with VEGF-C : structure, maturation process characterized by cleavage of a pre-protein into the mature form (Stacker et al., 1999) and, at least in human, the ability to bind both VEGFR-2 and VEGFR-3 (Achen et al., 1998) (while in mice, it seems to be able to bind only VEGFR-3 (Baldwin et al., 2001)). Over-expression of VEGF-D in tumor cells induces tumor angiogenesis and lymphangiogenesis (Stacker et al., 2001; Von Marschall et al., 2005) and in clinic, its over-expression is associated with metastatic dissemination and poor prognosis (Stacker et al., 2014). However, contrary to VEGF-C, its implication during lymphatic development remains unclear. Indeed, VEGF-D knockout mice are viable without any noticeable phenotype on blood or lymphatic endothelium (Baldwin et al., 2005), while VEGF-C/VEGF-D double knockout mice phenocopies the developmental phenotype of VEGF-C knockout mice (Haiko et al., 2008). This suggests that VEGF-D is not required for embryonic development mostly due to functional redundancy between the two factors, but can compensate for a VEGF-C deficiency as demonstrated in VEGF-$C^{+/-}$ mice where the lymphatic hypoplasia is suppressed by VEGF-D treatment (Baldwin et al., 2005). In parallel, other studies have found that VEGF-D could be important to ensure the development of specific lymphatic beds such as facial lymphatics in zebrafish (Bower et al., 2017b) or dermal lymphatics in mice (Paquet-Fifield et al., 2013). VEGF-D can thus be considered as a modulator of lymphangiogenesis lacking the raw potency of VEGF-C, but able to fine-tune the lymphatic vasculature in response to environmental signals. This has been illustrated at least in cancer where VEGF-D, by modulating prostaglandin levels can induce lymphatic vessel dilatation and thereby promote metastatic dissemination (Karnezis et al., 2012).Interestingly, VEGF-D displays also an

angiogenic activity (Jauhiainen et al., 2011) and could be useful in therapies where induction of both angiogenesis and lymphangiogenesis is needed such as coronary artery disease (CAD) (Rissanen et al., 2003). Currently, an Adenoviral intramyocardial vector using VEGF-D has given promising results in phase I clinical trial (Hartikainen et al., 2017).

4.1.4. VEGF-C and VEGF-D-based therapeutic strategies

Therapeutic strategies based on VEGF-C/VEGF-D viral vectors have been tested with the aim to provide new treatments for lymphedema. Lymphedema describes a progressive pathologic condition of the lymphatic system characterized by interstitial accumulation of protein-rich fluid and subsequent inflammation, adipose tissue hypertrophy, and fibrosis. The swelling and subsequent induration of the affected region can cause disfigurement, as well as decreased mobility and function. This condition remains difficult to treat and causes significant morbidity, both physical and psychologic, for patients (Grada and Phillips, 2017; Taghian et al., 2014). VEGF-D has been shown to display a potent lymphangiogenic effect when delivered through adenovirus-based vector, but it also induced a strong angiogenesis and increased vascular permeability and blood leakage (Stacker et al., 1999). This side effect results also in an accumulation of seroma fluid in a porcine model of lymphedema further excluding it as a good candidate for lymphedema therapy [215]. Aside from its lymphangiogenic properties, VEGF-C is also known to induce angiogenesis and increase blood vessels permeability [127, 216] and adenoviral VEGF-C overexpression in skin induce blood leakage in a murine model [217]. The extent of this adverse effect remains controversial as it has not been observed in experimental model set up in larger animal such as rabbit [218, 219] or porcine models [215, 220]. Based on these researches, a clinical trial using a VEGF-C adenoviral vector in combination with a surgical lymph node transfer for the treatment of patients with secondary lymphedema has been developed and is currently in phase 2 (ClinicalTrials.gov Identifier: NCT02994771 and NCT03658967).

4.2. Vascular endothelial growth factor receptors

Tyrosine kinase receptors, to which bind VEGFs, are composed of three domains: an extracellular domain for VEGF binding, a transmembrane domain and an intracellular domain with tyrosine kinase activity (Karaman et al., 2018). VEGF binding to the extracellular receptor domain promotes the activation of tyrosine kinase in the intracellular domain, which phosphorylates tyrosine residues, thus activating several intracellular signalling pathways. There are three types of VEGF receptors: VEGFR-1, VEGFR-2 and VEGFR-3. As both lymphangiogenesis and angiogenesis are mainly triggered by VEGFR-2 and VEGFR-3, the present review will focus on these two receptors and their partners.

4.2.1. VEGFR-2

VEGFR-2 [also known as kinase insert domain receptor (KDR) in human and fetal liver kinase 1 (Flk-1) in mouse] is expressed especially on endothelial cells of blood and lymphatic vessels but is also weakly expressed in hematopoietic cells, megakaryocytes, retinal progenitor cells, neurons, osteoblasts and pancreatic ductal cells (Shibuya, 2006). It has a molecular weight of 200–230 kDa and is the main receptor of VEGF-A showing a much greater affinity for it than for VEGF-C and –D (Takahashi and Shibuya, 2005). VEGF-A/VEGFR-2 signalling is therefore essential for the normal course of vasculogenesis during the embryonic development as its inhibition results in embryonic death on days 8–9, due to a failure of development and organization of the blood vascular network (Olsson et al., 2006). When VEGF-A binds to the extracellular domain of VEGFR-2, the receptor undergoes homodimerization and autophosphorylation of tyrosine residues, in particular the tyrosine residues 951 and 1059, which are essential to trigger VEGF-A-induced migration and proliferation (Zeng et al., 2001), by activating signalling pathways such as: Ras/Raf/ERK/MAPK and phospholipase-Cγ (PLCγ)/protein kinase C (PKC) in BECs (Cebe-Suarez et al., 2006). It also plays an anti-apoptotic role through the activation of PI3K/Akt pathway. Taken together, these data clearly show that the interaction between VEGF-A and VEGFR-2 homodimers is a key pathway to promote angiogenesis.

This receptor is also responsible for lymphangiogenic properties of VEGF-A. Indeed, VEGF-A nearly only acts through VEGFR-2 homodimerization to trigger its effects on LECs contrary to VEGF-C, which relies heavily on VEGFR-3. Indeed, while VEGF-C is able to bind VEGFR-2 without requiring the presence of VEGFR-3 (Joukov et al., 1998), VEGFR-2 homodimerization is not induced upon VEGF-C stimulation in cells expressing both VEGFR-2 and VEGFR-3 : instead, only VEGFR-3 homodimers and VEGFR-2/R-3 heterodimers (see below for more details about receptors heterodimerization) has been observed upon VEGF-C stimulation (Nilsson et al., 2010). These data highlight that VEGFR-2 homodimerization is a specific feature of VEGF-A stimulation. Once linked to VEGFR-2, VEGF-A induces a rapid and strong activation of ERK1/2 pathway. VEGF-A signalling on LECs seems to be straightforward as the other main pathways such as phosphoinositide 3-kinase/AKT pathway were only weakly induced (Deng et al., 2015).

4.2.2. VEGFR-3

VEGFR-3 or Fms-like tyrosine kinase 4 (Flt-4) (Wigle and Oliver, 1999) has a molecular weight of 195 kDa. Contrary to VEGFR-2, which is mainly involved in angiogenesis, VEGFR-3 plays an important role in the lymphatic vasculature development during embryogenesis. VEGFR3 expression is initiated at embryonic day 8 in blood vessels, but later, its expression becomes restricted to the lymphatic vasculature. Initially, VEGFR3 expression is induced in a specific subset of endothelial cells, i.e. lymphendothelial precursors, in the cardinal vein during development. Then, at E10.5, lymphendothelial precursors that express VEGFR3 bud off and migrate away from the embryonic cardinal vein towards a gradient of VEGFC, which is produced by nearby mesenchymal cells. Migrating LECs subsequently assemble into lymph sacs that extend to be the starting point of the lymphatic system development (Yang and Oliver, 2014). Later in development, VEGFR3 expression is downregulated on vascular endothelial cells except during angiogenesis, where it becomes induced and expressed at higher levels (Tammela et al., 2008). It is also involved in the formation of new lymphatic vessels in the adult life influencing the proliferation, migration, tubulogenesis and survival of LECs (Karaman et al., 2018). In addition to the lymphatic endothelium, VEGFR-3 expression has been reported on osteoblasts (Orlandini et al., 2006), macrophages (Alishekevitz et al., 2016) and neural progenitors(Han et al., 2015). Contrary to VEGFR-2, which mainly binds VEGF-A, VEGFR-3 has a much higher affinity for VEGF-C and VEGF-D. The binding of these two factors to the extracellular domain of VEGFR-3 induces its homodimerization and autophosphorylation to trigger lymphangiogenesis. The extracellular domain of VEGFR-3 consists of seven Ig homology domains (D1-7) whose roles have been characterized in details (Leppanen et al., 2013). Briefly, D1-3 are responsible for ligand binding while membrane-proximal D4–7 are involved in structural rearrangements essential for receptor dimerization and activation. In particular, an homotypic interaction between D5 and D7 has been shown to be required for VEGF-C-mediated activation of VEGFR-3 (Leppanen et al., 2013). Interestingly, in addition to being required for VEGFR-3 maturation, D5 is implicated in both VEGFR-3 homodimerization and heterodimerization (see below) as antibodies designed to bind this domain effectively inhibit both types of VEGFR-3 dimerization (Tvorogov et al., 2010). While VEGF-A/VEGFR-2 signalling in LECs is pretty straightforward, heavily relying on ERK phosphorylation, VEGF-C/VEGFR-3 displays a more polyvalent signal transduction, activating several pathways such as : ERK (however to a lesser extent a slower kinetics than what is observed with VEGFA/VEGFR-2), AKT and c-JUN N-terminal kinase-1/2 (JNK1/2) (Deng et al., 2015; Salameh et al.,

2005)

4.2.3. VEGFR-2/VEGFR-3 heterodimerization

VEGFR-2 and VEGFR-3 can also heterodimerize, leading to different signalling outcomes. The function of VEGFR-2/VEGFR-3 heterodimers in BECs has been extensively studied during angiogenesis. In fact, VEGFR-3 is not expressed in unstimulated BECs but become induced during angiogenesis forming heterodimers with VEGFR-2. Heterodimers are prominent in tip cells of angiogenic sprouts allowing both VEGF-A and VEGF-C to regulate angiogenic sprouting (Nilsson et al., 2010). VEGFR-2 or VEGFR-3 blockade prevents heterodimer formation and reduced angiogenesis (Nilsson et al., 2010). Interestingly, in BEC, VEGF-A elicits a robust VEGFR-2 homodimerization associated with a weak VEGFR-2/R-3 heterodimerization. In the opposite, VEGF-C stimulation triggers both a strong VEGFR-3 homodimerization and VEGFR-2/R-3 heterodimerization, and a marginal VEGFR-2 homodimerization. In LEC, the role of VEGFR-2/VEGFR-3 heterodimers is anticipated but still poorly documented. Interestingly, it has been demonstrated that five tyrosine phosphorylation sites in the carboxyl-terminal tail of VEGFR-3 can be phosphorylated upon VEGF-C stimulation in VEGFR-3/R-3 homodimers. In sharp contrast, only three of those sites are activated in VEGFR-2/R-3 heterodimers suggesting different outcomes in signal transduction (Dixelius et al., 2003). A cooperative signalling of VEGFR-2 and -3 appears necessary for lymphatic migration and proliferation, while VEGFR-3 is redundant with VEGFR-2 for LEC organization into functional capillaries, in an adult model of lymphangiogenesis during skin regeneration (Goldman et al., 2007). The importance of VEGFR-2/R-3 heterodimers in lymphatic vessel formation is supported by distinct effects exerted by wild type VEGF-C and a mutated form (VEGF-C156S), which only binds VEGFR-3 homodimers. Indeed, in a porcine model of lymphedema, VEGF-C induces a superior lymphangiogenic response than its mutated counterpart, without displaying any vascular side effects (Dixelius et al., 2003; Goldman et al., 2007). Therefore, VEGF-C interacting with both homodimers and heterodimers is the preferred pro-lymphangiogenic agent for lymphedema treatment (Visuri et al., 2015; Tervala et al., 2015). The regulation of VEGFR-2/R-3 heterodimers during lymphangiogenesis involves the urokinase plasminogen activator receptor-associated protein, uPARAP/Endo180 (MRC2 gene) recently identified as a new regulator of lymphatic growth (Durre et al., 2018). Genetic deletion of uPARAP is associated with a hyperbranched lymphatic vasculature characterized by an excessive sprouting. Interestingly, this effect has been seen only when lymphatic growth was induced by VEGF-C, while uPARAP status did not affect lymphangiogenesis triggered by either VEGF-A or VEGF-C156S excluding a contribution of VEGFR-2 or VEGFR-3 homodimers. For the first time, this study provides evidence that uPARAP is able to bind both VEGFR-2 and VEGFR-3 and restricts their heterodimerization upon VEGF-C stimulation. uPARAP silencing led to an excessive level of VEGFR-2/VEGFR-3 heterodimers leading to the inactivation of CRK-II adaptor molecule by its VEGFR-2-mediated phosphorylation. Crk-II is thus less recruited by VEGFR-3 reducing thereby JNK pathway activation. Importantly, uPARAP downregulation resulted in an over-activation of Rac1 that impaired the directional migration of LECs in a VEGF-C gradient in vitro and resulted in an hyperbranched lymphatic vasculature in vivo. A pharmacological inhibitor of Rac-1 used in uPARAP KO mice led to a lymphatic morphogenesis similar to that observed in WT mice (Durre et al., 2018). Interestingly, these effects were seen only in lymphatics while blood vasculature remains unaffected by the uPARAP status. These findings shed light on the importance of VEGFR-2/VEGFR-3 heterodimerization during lymphangiogenesis and further underline the differences between the lymphangiogenic and angiogenic processes.

4.2.4. Neuropilins

Numerous co-receptors have also been described for VEGFRs. They add another level of regulation of VEGFR signalling by modulating its time course and amplitude. Among others, members of the neuropilin family like the semaphorin receptor neuropilin 1 and 2 (NRP1, NRP2) have been identified as key VEGFRs partners. Both of them are transmembrane molecules with a short cytoplasmic tail, which lacks intrinsic enzymatic activity and have a low molecular weight of 120–135 kDa (Parker et al., 2012). NRP1 was shown to bind VEGF-A and be an essential part of the VEGF-A/VEGFR-2 complex. NRP1 regulates VEGFR-2 signalling by acting on the ERK1/2 phosphorylation upon VEGF-A stimulation in BECs, thus strengthening the angiogenic process (Teran and Nugent, 2015; Hoeben et al., 2004). In addition to the formation of new vessels by acting on proliferation and migration of BECs, NRP1 also increases VEGF-A-mediated vascular permeability through its interaction with VEGFR-2 (Fantin et al., 2017) giving it a particularly important role in the blood vasculature.

NRP2 is the lymphatic counterpart of NRP1, binding VEGF-C (Parker et al., 2012) and regulating the VEGF-C/VEGFR-3 axis in lymphatics (Xu et al., 2010). Given that NRP2 can also interact with VEGFR-2 (Favier et al., 2006), one could argue that NRP2 is not really a lymphatic-related regulator but share the same role in both blood and lymphatic systems. However, NRP2 is far more expressed in lymphatic vasculature than in the blood one where it is only observed in veins (Olsson et al., 2006; Herzog et al., 2001). In addition, in vivo studies have shown that NRP1$^{-/-}$ mice died around embryonic day E12.5 to E13.5, displaying defects in blood vascularization (Kawasaki et al., 1999) while NRP2-deficient mice displayed normal development of blood vessels, as well as of larger collecting lymphatic vessels. However they exhibit a severe reduction in small lymphatic vessels and capillaries (Yuan et al., 2002). Interestingly, when NRP2 and VEGFR-2 or NRP2 and VEGFR-3 are both heterozygously deleted, a lymphatic defect is only observed in the double-heterozygote *nrp2vegfr3* mice. While not completely excluding a role for NRP2 in BECs, the data available currently highlight that while NRP1 is strongly implicated in the regulation of blood vessels formation and functionality, NRP2 is a key component of the lymphatic system (Fig. 2).

5. Concluding remarks

Even if they share many properties, both lymphatic and blood systems have their own features. The data presented in this review demonstrate that despite many similarities in regard to the signalling pathways implicated in the regulation of the two vasculatures, advances in angiogenesis cannot be directly translated to the lymphangiogenic field. Given the tremendous number of pathologies where an imbalance of angiogenesis or lymphangiogenesis is implicated, it is crucial to pay attention to the specificities of each vascular network in order to design more efficient therapies in vascular-related diseases either to act on only one of the two vascular systems or to target both of them concomitantly. The clinical interest to target VEGF-C/VEGFR-3 pathway is under study, and characterizing other pathways involved in the regulation of LEC functions is mandatory to provide additional opportunities to treat diseases associated to excessive lymphangiogenesis. In addition, appropriate blood and lymphatic vessel formation is essential for tissue regeneration covering wound healing to engineered organ transplantation (Alderfer et al., 2018). Whether vessel formation has to be stimulated or blocked, new insights into LEC and BEC specificities are required to design efficient therapies. Advances in lineage tracing tools and single-cell RNA-Sequencing approaches will provide important high-resolution mapping of LEC and BEC heterogeneity under various pathological conditions.

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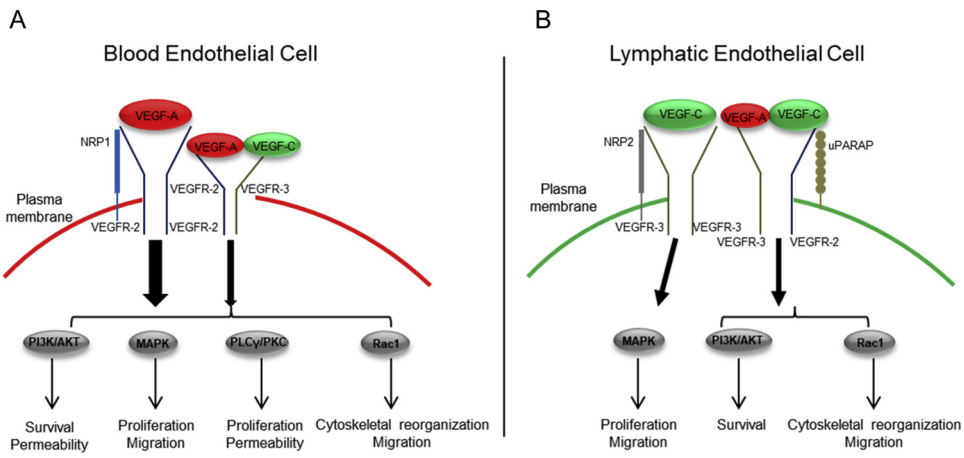

Fig. 2. Schematic representation of the main signalling pathways.
A: Blood endothelial cells heavily rely on VEGF-A for proliferation, migration and survival. Most of the VEGF-A effects are triggered through its association with VEGFR-2 homodimers though VEGFR-2/VEGFR-3 heterodimers play also a role during sprouting. B: VEGF-C is the most potent stimulating factor for lymphatic endothelial cells and is able to act both with VEGFR-3 homodimers and VEGFR-2/VEGFR-3 heterodimers to promote LECs survival, proliferation and migration.

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