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Cell-autonomous and paracrine mechanisms underlying *Pik3ca*-driven vascular malformations

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Abstract

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Vascular malformation is a benign overgrowth of blood or lymphatic vessels leading to life-threatening consequences for affected patients. Activating mutations in the TIE2 receptor cause the majority of venous malformations (VMs), while somatic activating mutations in *PIK3CA*, leading to the overactivation of the PI3K-AKT pathway, cause both VMs and lymphatic malformations (LMs). Although molecular inhibitors targeting the PI3K-AKT-mTOR pathway, such as rapamycin, have shown beneficial effects, they are not curative. This thesis aimed to explore the endothelial cell-autonomous and paracrine mechanisms underlying *Pik3ca*-driven pathological vascular growth to identify a rationale for improved and curative therapies for vascular malformations.

In **Paper I**, we reported that one of the most common causative mutations, $PIK3CA^{H1047R}$, gives rise to two distinct LM subtypes known as macrocystic and microcystic LM in humans. Using a transgenic mouse model with temporally controlled LEC-specific activation of $Pik3ca^{H1047R}$, we found that the growth of microcystic LM is dependent on both the upstream pro-lymphangiogenic VEGF-C-VEGFR3 and the downstream AKT-mTOR signalling. Combination treatment targeting both signalling pathways led to effective inhibition of lesion growth in mice, suggesting a novel therapeutic approach for LM patients. In **Paper II**, we explored further the endothelial cell-autonomous and paracrine mechanisms underlying microcystic LM growth in mice. Using single-cell RNA sequencing, we identified a new immune-interacting subtype of dermal lymphatic capillary endothelial cells, termed iLECs. We showed that in *Pik3ca* mutant mice, iLECs produce factors that recruit pro-lymphangiogenic VEGF-C-producing macrophages. Macrophage depletion, inhibition of their recruitment, and anti-inflammatory COX-2 treatment resulted in decreased lymphatic growth, indicating a critical role of paracrine signalling between iLECs and immune cells in the pathogenesis of microcystic LM. In **Paper III,** we described distinct lymphatic vessel responses to oncogenic PI3K activation in different organs. We observed that while lymphatic vessels in the skin form microcystic LM through vessel sprouting, in certain other organs, they form large cysts reminiscent of macrocystic LM. Finally, we used mice with a BEC-specific activation of $Pik3ca^{H1047R}$ to compare disease mechanisms in VM to those in LM in Paper II and to focus further on the former in **Paper IV**.

Keywords: PI3K, PI3KCA, Venous malformations, H1047R

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“Somewhere, something incredible is waiting to be known.”
Carl Sagan

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Martinez-Corral I #, Zhang Y #, **Petkova M**, Ortsäter H, Sjöberg S, Castillo SD, Brouillard P, Libbrecht L, Saur D, Graupera M, Alitalo K, Boon L, Vikkula M, Mäkinen T. (2020) Blockade of VEGF-C signaling inhibits lymphatic malformations driven by oncogenic *PIK3CA* mutation. *Nat Commun* 11(1):2869

II. **Petkova M**, Kraft M, Stritt S, Martinez-Corral I, Ortsäter H, Vanlandewijck M, Jakic B, Baselga E, Castillo SD, Graupera M, Betsholtz C, Mäkinen T. (2023) Immune-interacting lymphatic endothelial subtype at capillary terminals drives lymphatic malformation. *J Exp Med* 220 (4): e20220741 (cover image Vol220)

III. **Petkova M** #, Schoofs H #, Testroet F, Matinez-Corral I, Mäkinen T. (2023) Organ-specific mechanisms of *Pik3ca*-driven lymphatic malformation. *Manuscript.*

IV. Kraft M, Schoofs H #, **Petkova M** #, Andrade J, Grosso AR, Benedito R, Potente M, Mäkinen T. (2023) Venous-specific autocrine signalling promotes *PIK3CA*-driven vascular malformations. *Manuscript.*

# Contributed equally

Contents

Abbreviations

αSMC	Alpha-smooth muscle actin
ACKR2	Atypical chemokine receptor 2
ANGPT1/2	Angiopoietin-1/2
BEC	Blood endothelial cell
CCL2 (MCP-1)	CC-chemokine ligand 2, monocyte chemoattractant 1
CCL21	C-C motif ligand 21
CCM	Cerebral Cavernous Malformation
CCR2	CC-chemokine receptor 2
CD31	Cluster of differentiation 31 (PECAM-1)
CD45	Protein tyrosine phosphatase, receptor type, C (PTPRC)
CSF1	Colony-stimulating factor
DC	Dendritic cell
ECM	Extracellular matrix
EC	Endothelial cell
EMCN	Endomucin, marker for venous and blood capillary ECs
FGF2	Fibroblast Growth Factor 2
FOXO1	Forkhead box protein O1
INF-γ	Interferon-gamma
LEC	Lymphatic endothelial cell
LM	Lymphatic malformation
LPS	Lipopolysaccharide
LYVE-1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1
MHCII	Major histocompatibility complex molecules class II
mTOR	Mammalian target of rapamycin
NRP2	Neuropilin 2
PDPN	Podoplanin
PI3K	Phosphoinositide 3-kinase
PIK3CA	Gene encoding PI3K Catalytic Subunit Alpha
PROS	*PIK3CA*-overgrowth spectrum
PROX1	Prospero homeobox protein1
PTX3	Pentraxin 3, TNF-stimulated gene 14 (TSG-14)
TAM	Tumour-associated macrophage
TEK	Gene encoding TIE2 receptor protein
TLO	Tertiary lymphoid organ
TNF	Tumour necrosis factor α
VEGFR (1/2/3)	Vascular endothelial growth factor receptor
VEGF (-A/B/C/D)	Vascular endothelial growth factor
VM	Venous malformation

Introduction

Blood and lymphatic vascular system

Organisation and function

The circulatory system of mammals consists of two interconnected hierarchical networks, the blood and lymphatic vasculatures, that cooperatively maintain tissue survival, growth and homeostasis. The blood vascular system transports gas, nutrients, metabolites, and other signalling molecules to the tissue. In contrast, the lymphatic system returns the extravasated fluid from the tissue to the blood circulation (1). Besides maintaining fluid balance, the lymphatic system transports immune cells and peripheral antigens to lymph nodes. Furthermore, lymphatic vessels possess organ- and tissue-specific functions such as lipid absorption, cholesterol clearance, electrolyte homeostasis, stem cell regeneration, and immune modulation (2–6).

The different functions of the two systems are mirrored by their distinct structural organisation. The blood vascular system forms a closed network of arteries, veins and connecting capillaries. Oxygenated blood flows from the heart through the aorta into large arteries that branch into smaller arterioles and further to the capillary network. The venules drain the blood from the capillaries into bigger veins to return it to the heart. Additionally, the blood passes the pulmonary artery to the lungs, where it gets re-oxygenized. In contrast, the lymphatic system is a blind-ended, unidirectional network that consists of initial lymphatic vessels, called lymphatic capillaries, that drain the interstitial fluid from the tissue to progressively larger pre-collecting and collecting vessels, where the fluid is termed lymph **(Figure 1)**. Further, the lymph travels through the lymph nodes into the thoracic and right collecting ducts before returning to the blood circulation (1).

Heterogeneity in vessel morphology

The vessels of both systems are composed of endothelial cells (ECs), forming the inner layer specialised in sensing and responding to the changes both in the intra- and extravascular environment. Despite their shared transport role and common mesodermal origin, vessels have distinct functions due to the high heterogeneity of ECs in structure and gene/protein expression (1,7,8). This heterogeneity can manifest in distinct tissue-specific morphological

features reflecting the organ specification and function of vessels and ECs. For instance, vessels are covered by a basement membrane, which varies from a thick elastic lamina in the arteries to a thin discontinuous membrane in lymphatic capillaries. Furthermore, the **mural cell coverage** of vessels also differs within the vascular tree. Large arteries contain vascular smooth muscle cells, aiding their function in regulating vascular tone. Conversely, venules and blood capillaries are covered by pericytes that allow immune cell extravasation. Lymphatic vessels also have a distinct mural cell coverage. While collecting vessels have a smooth muscle cell layer, aiding lymph pumping, capillaries lack mural cells.

Other essential features that vary between the vessel types are **cell shape and cell junctions,** that reflect their distinct purposes. ECs in blood vessels form a monolayer supported by tight, adherence and gap junctions, which can differ between vascular beds. For instance, brain microvascular ECs have specialised tight and adherence junctions, required for a low level of transcytosis and maintenance of the so-called blood-brain barrier (9). On the contrary, sinusoidal ECs in the liver exhibit specialised intercellular junctional complexes and transcellular pores, suited for high permeability (10). In addition, the shape of ECs varies between arteries and veins. While arterial ECs are elongated and narrow, aligned in the direction of blood flow, venous ECs are wider and less extended. Additionally, the large blood vessels and some of the capillaries possess continuous basement membrane. Capillary lymphatic ECs (LECs) exhibit button-like intercellular junctions, oak-leaf shape and discontinuous basement membrane, all facilitating the uptake of fluid and soluble molecules. In contrast, lymphatic collecting vessels have continuous zipper-like junctions that prevent leakage and aid in transporting immune cells and antigens to the lymph nodes. To prevent backflow, the lymphatic collecting vessels, similar to the veins, possess unique morphological structures called luminal valves (**Figure 1**) (1). The unidirectional movement of lymph is maintained not only by the valves but also by the muscle contractions of the collecting vessels, skeletal muscle movements, respiratory action and the arterial pulse of the body (6,11).

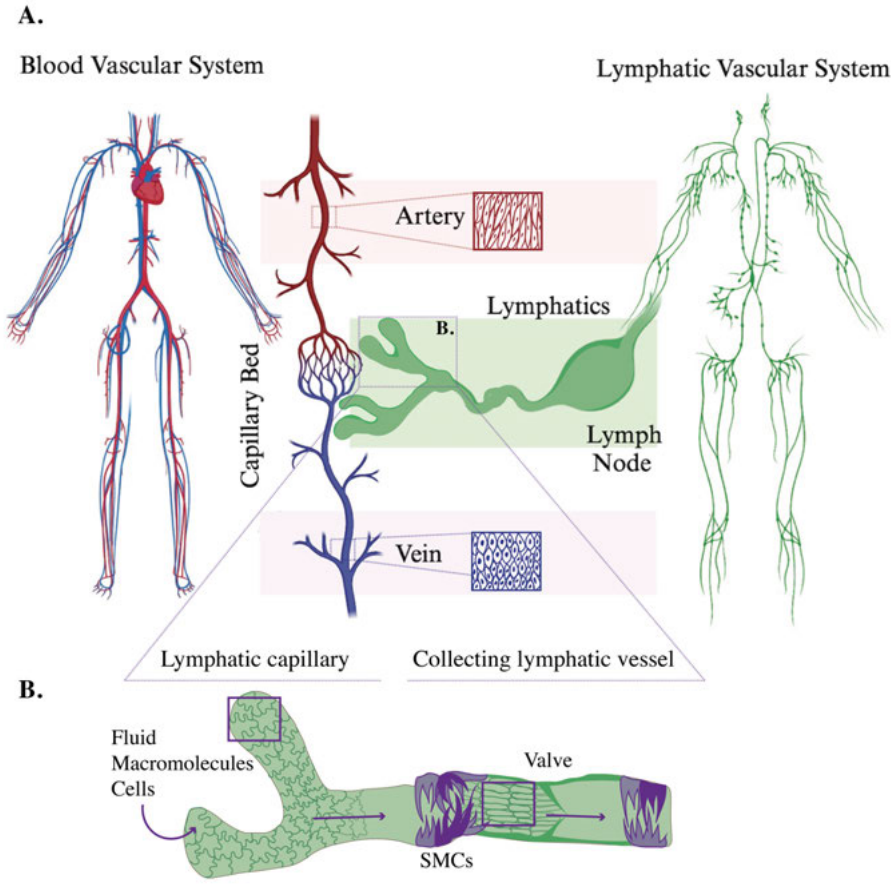


Figure 1. Organisation of the vascular system. The circulatory system of mammals consists of a closed blood vascular system and a unidirectional blind-ended lymphatic vascular system. The blood vascular system forms a network of arteries, veins and connecting capillaries. Boxes show distinct cell shape, where the arterial ECs are elongated and venous are wider (**A**). The lymphatic system includes lymph nodes, lymphatic capillaries, which drain fluid, macromolecules and cells from the tissues, and collecting lymphatic vessels with distinct morphological features, such as valves and smooth muscle cell (SMC) coverage. Boxes show oak-leaf cell shape in the capillary lymphatics and elongated shape in the collecting lymphatic vessels (**B**). The circulatory systems in (**A**) were created with Biorender.com. (**B**) redrawn and adapted from (12)

Organ-specific heterogeneity

In addition to vessel-specific heterogeneity, ECs in different organs possess remarkable plasticity in order to adapt to the diverse functions and molecular signalling of the specific tissue environment (6,13). Recent studies using single-cell transcriptomics have both confirmed the organ-specific heterogeneity and also revealed an intra-organ EC diversity across the tissue (7,8,14,15). Intriguingly, a gradual change in EC transcriptional profile has been documented, called arterial-venous transcriptional continuum or zonation. The transcriptional zonation has been described in several organs, both in mice and humans. It likely mimics a gradual transition in the phenotype of ECs when they change their vessel identity (16,17). A better understanding of the organ- and vessel-specific features of the specialised vascular beds is of crucial importance in revealing the mechanism behind vascular-related pathologies. Here, the unique features of the vascular beds studied in this PhD work, including the skin and mesentery, will be discussed in more detail.

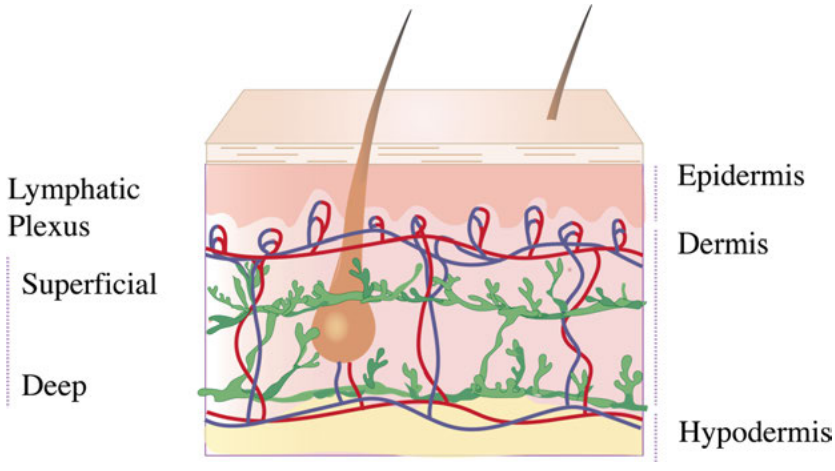


Figure 2. Skin vascular bed. Schematic representation of a cross-section of the skin, showing the main skin layers: epidermis, dermis, and hypodermis. The blood and lymphatic vascular systems are organised into two horizontal plexuses. The superficial and deep lymphatic plexuses have been marked. Lymphatics are presented in green, veins in blue and arteries in red.

As the body's first physical and physiological barrier against pathogens, the skin consists of an avascular durable epidermis and an inner vascularised dermis. The **skin vasculature** in mice and humans shares a similar morphology, organised into two horizontal plexuses connected by vertical dermal capillary loops (18). Similarly, adjacent to the blood vessels, the lymphatic vasculature also forms two plexuses: a superficial layer comprised of lymphatic capillaries and a deeper one consisting of collecting vessels (**Figure 2**). Interestingly, while blood microvessels are located immediately beneath the epidermis, the

superficial lymphatic vessels are situated deeper within the dermis. From the superficial plexus, lymphatic capillaries drain vertically into a series of larger lymphatic vessels to the deeper dermis and further to the subcutaneous tissue towards the tissue-draining lymph node. Both draining venules and deeper lymphatic vessels contain numerous valves (18).

The superficial lymphatic vessel plexus described above is similar throughout the skin of the human body. Yet, some areas, such as the fingers, the palm, and the scrotum, have a more abundant lymphatic network (18). Intriguingly, in both mice and humans, lymphatic vessel density in the skin is notably higher than in other organs, which may be due to the role of dermal lymphatic vessels in immune surveillance against foreign antigens and microorganisms after skin damage (18,19). It may also be due to a specialised regenerative demand in the skin since lymphatic vessels were recently found to provide a stem cell niche for hair follicle regeneration both in mice and humans (20,21).

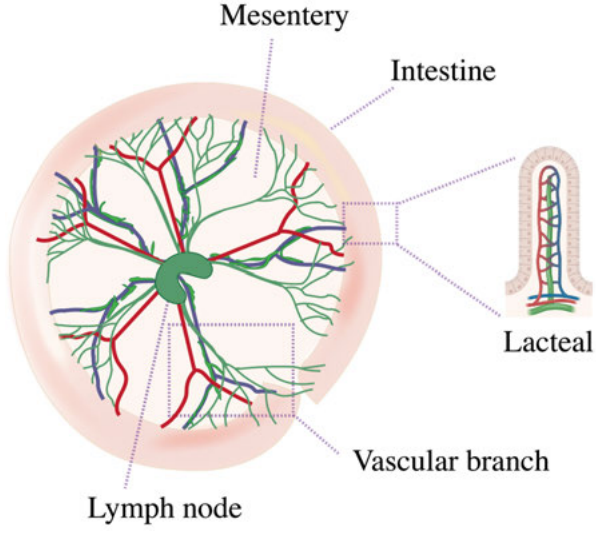


Figure 3. Vascular bed in the mesentery. Schematic representation of mesentery from the small intestine. Blood within the mesentery circulates in a complex network of arteries and veins that connect to the deep organs. Lymph flows from the intestinal lacteals to collecting vessels, reaching the mesenteric lymph node. A separate lymphatic capillary network drains the mesenteric tissue directly to the mediastinal lymph nodes. Lymphatics are presented in green, veins in blue and arteries in red. Box points out to a vascular branch of the mesentery, which is often preferred for imaging representation. The lacteal has been adapted from Biorender.com.

While the skin covers the whole body, providing protection from the outside environment, the **mesentery** is localised in the peritoneal cavity, where all abdominal digestive organs develop and remain connected to it. Besides preventing tissue adhesion, the mesentery is involved in transporting fluid and cells and regulating the immune response (22). The mesentery is formed by

mesothelium and connective tissue and has a complex vascular network. The primary blood supply is provided by the superior and inferior mesenteric arteries, which branch into smaller arterioles. The complex venous system consists of gastric, splenic and hepatic veins and venules, draining blood from distinct inner organs (3,23). Previous studies showed that intestinal lymph flows from the lymphatic capillaries of the intestinal villi, called lacteals, and crypts to lymphatic collecting vessels, confined to the mesentery, before reaching the mesenteric lymph nodes and further the thoracic duct and blood circulation (3). In addition, a recent study showed a separate mesenteric capillary lymphatic network that drains directly to the mediastinal lymph nodes (**Figure 3**) (24).

Morphological or functional defects of the blood and lymphatic system have been linked to various pathological conditions, such as lymphedema, cardiovascular diseases, obesity, inflammation, fibrosis, neurogenerative diseases, and cancer (6,25). Genetically modified mice can reproduce vascular phenotypes in various pathologies, such as lymphedema, cancer and metastasis, and vascular malformations. (18,26–30). However, it is important to note that while the main structure of the vascular system is conserved across different species, there may be specific functional and molecular differences, as recently highlighted in relation to the lymph node vasculature (8,31).

Regulation of blood and lymphatic vasculatures

Angiogenesis and VEGF signalling

The blood and lymphatic vasculatures not only differ in morphology and function but also rely on distinct mechanisms for growth and remodelling (1). In mice, the first blood endothelial progenitor cells differentiate from mesoderm, acquire venous or arterial fate and build a primitive vessel network in a process called **vasculogenesis**. This process is controlled by factors such as fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 4 (BMP4), which are responsible for blood EC (BEC) specification and differentiation. Furthermore, BEC proliferation and survival are regulated by vascular endothelial growth factor A (VEGF-A), an essential factor for their development (1,32). Further blood vessel growth, both during embryonic development and in postnatal tissue expansion and pathology, is achieved through the formation of new vessels from existing ones, a process known as **angiogenesis** (33). During this process, vessels sprout and branch to form a primitive vascular network, which is further remodelled into a functional vascular system. Tip cells, located at the leading edge of sprouting blood vessels, sense growth factor gradients and guide the newly formed sprouts in the correct direction. Stalk cells, positioned behind tip cells, proliferate, stabilise the vessel and form a lumen (1,34).

Angiogenesis is a dynamic process, often initiated by tissue hypoxia and maintained by a balance between pro- and anti-angiogenic factors. The primary pro-angiogenic factors include vascular endothelial growth factors (**VEGFs**) and their receptor tyrosine kinases (**VEGFRs**). The family of VEGFs contains VEGF-A (known as VEGF), VEGF-B, VEGF-C, VEGF-D and placental growth factor. VEGFs bind to the extracellular domains of VEGFRs, including VEGFR1, VEGFR2 and VEGFR3, leading to receptor dimerisation and phosphorylation and triggering downstream signal transduction (35). The key angiogenic factor VEGF-A binds to VEGFR1 and VEGFR2 on BECs to initiate tip cell formation, migration, and proliferation (32,36). The importance of VEGFR1 and VEGFR2 in blood vascular development is demonstrated by the lethality of mice that lack *Vegfr1* or *Vegfr2*, leading to either excessive endothelial cell proliferation and disassembly or impaired vessel development, respectively (37,38). While BECs with high expression of VEGFR2 and VEGFR3, together with NOTCH ligand Delta-like protein 4 (DLL4), become tip cells, the remaining cells called stalk cells activate NOTCH1 signalling and upregulate VEGFR1, acting as a decoy receptor for VEGF-A (**Figure 4**) (39,40). The transition between tip and stalk cell identities is a complex and dynamic process which depends not only on VEGF-A-VEGFR- and DLL4-NOTCH1-signalling but also on neuropilin 1 (NRP1) and transforming growth factor- β (TGFβ)–BMP signalling (1,41,42).

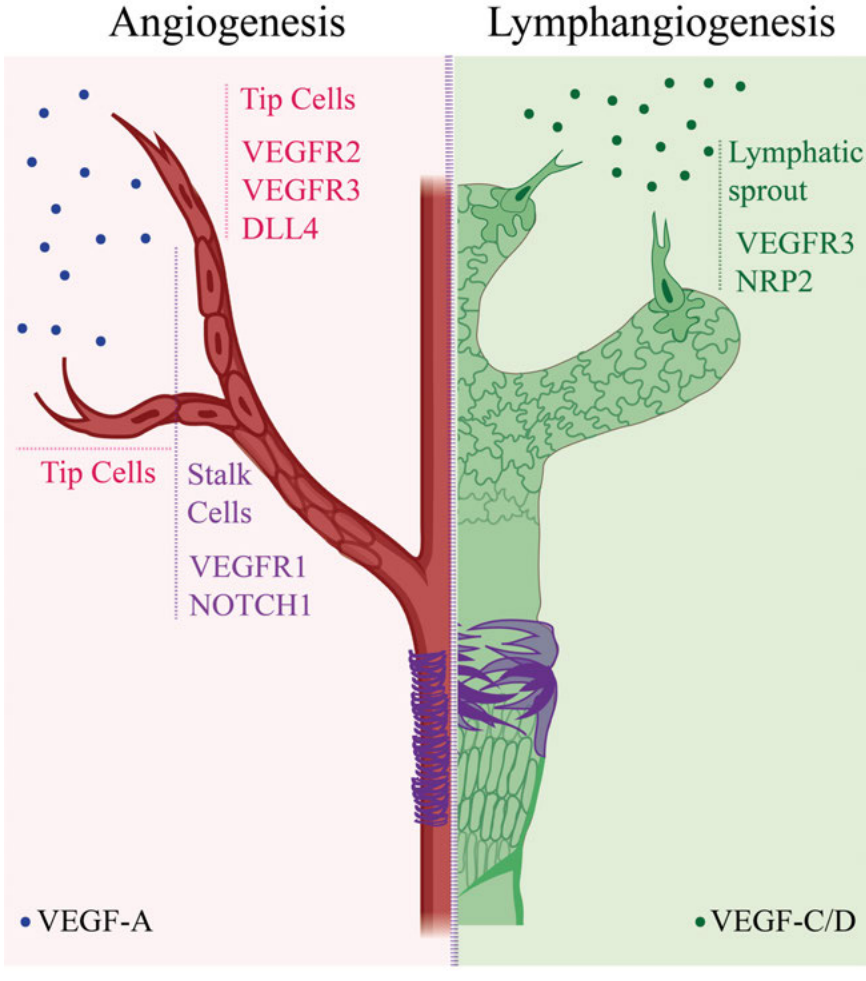


Figure 4. Regulation of vascular growth. New blood vessels are formed from preexisting ones through sprouting, a dynamic process called angiogenesis. Angiogenesis is dependent on the primary pro-angiogenic factors - vascular endothelial growth factors (VEGFs) and their receptor tyrosine kinases (VEGFRs). Tip cells (VEGFR2$^+$, VEGFR3$^+$, DLL4$^+$) are located at the leading edge of sprouting blood vessels and VEGF-A gradients guide the newly formed sprouts. Stalk cells (VEGFR1$^+$, NOTCH1$^+$) are positioned behind the tip cells and proliferate, stabilising the vessel and forming a lumen. The growth of lymphatic vessels from pre-existing ones is called lymphangiogenesis, driven by pro-lymphangiogenic factors VEGF-C and D through their tyrosine kinase receptor VEGFR3 and co-receptor NRP2.

Lymphangiogenesis and VEGF-C-VEGFR3 signalling

Lymphatic development begins after functional blood circulation is established through the differentiation and migration of LECs from the cardinal vein (43). In addition, in certain organs, such as the skin and the mesentery, lymphatic vessels develop from progenitors of distinct non-venous origin (44–47). While the LEC fate regulator prospero homeobox protein1 (PROX1) reprograms the transcriptional profile of ECs from venous to lymphatic identity, the migration of the $PROX1^+$ LECs from the veins is dependent on the crucial growth factor for LECs - VEGF-C (48–50). *Vegfc*-deficient embryos exhibit lethality caused by the absence of lymphatic vasculature. In these embryos, $PROX1^+$ LECs do not migrate towards VEGF-C produced in the surrounding mesenchyme and thus remain unable to form sprouts (50). VEGF-C is also required for the growth of lymphatic vessels from pre-existing ones, a process known as **lymphangiogenesis**. This process occurs not only during embryogenesis but also during postnatal development and pathogenesis (6,25).

Lymphangiogenesis is primarily driven by signalling between VEGF-C and its tyrosine kinase receptor VEGFR3 and co-receptor neuropilin 2 (NRP2), promoting LEC migration, proliferation and vessel sprouting (**Figure 4**) (50–52). Inhibition of VEGFR3 signalling during embryonic or postnatal development leads to lymphatic vessel regression and lymphedema (52,53). Intriguingly, the second VEGFR3 ligand, VEGF-D, is dispensable for lymphatic development since mice lacking *Vegfd* exhibit a normal lymphatic vasculature (54). However, overexpression of either VEGF-C or VEGF-D in the murine skin induces sprouting of the cutaneous lymphatic vessels (55,56). The pro-lymphangiogenic activity of both proteins is controlled by proteolytic processing. While VEGF-C is proteolytically cleaved by ADAMTS3-CCBE1 complex during lymphatic development, VEGF-D is activated in adult skin by inflammatory proteases during wound healing. These distinct proteolytic mechanisms suggest different biological roles of the two growth factors (57).

Essential pathways interacting with VEGF-C signalling

The VEGF-C-VEGFR3 signalling cascade interacts with a number of other critical pathways. For instance, VEGF-C-VEGFR3 is an upstream regulator of DLL4-NOTCH1 signalling in the lymphatic vasculature (52). The embryonic lymphatic plexus remodels into a functional lymphatic network during late embryonic and early postnatal development, dependent on the VEGF-C-VEGFR3 pathway. Inhibition of VEGF-C signalling blocks lymphangiogenesis during this stage but does not affect the lymphatic network in the adult skin, which relies on DLL4-NOTCH1 signalling (53,58,59). Intriguingly, NOTCH1 inhibition leads to reduced lymphatic sprouting in neonatal mice while triggering lymphatic hyperplasia in adult mice (59,60). Additional

research shows that although the VEGF-C-VEGFR3 pathway is dispensable for the maintenance of lymphatic vessels in most adult organs, it is required in the lacteals and meningeal lymphatic vessels (58,61,62).

VEGF-C/D-VEGFR3 pathway has a significant role also in inflammation-induced lymphangiogenesis during adulthood in numerous pathologies such as cardiovascular diseases, chronic inflammatory diseases, cancer, and metastatic spread (63–66). During pathological lymphangiogenesis, VEGF-C is often produced by activated immune cells, including macrophages, tumour-associated fibroblasts and mutated tumour cells (64,67,68). Other pathways, such as VEGF-A/VEGFR2, Tumour necrosis factor α (TNF-α), NF-kB, COX-2, and prostaglandin E2 receptor signalling are also involved in the pathological lymphatic overgrowth (65,66,69). VEGF-C can also bind to VEGFR2, which is highly expressed in LECs in vivo. The primary angiogenic VEGFR2 ligand, VEGF-A, can promote LEC proliferation and migration in vitro (70–72). However, it still needs to be clarified if VEGF-A/VEGFR2 signalling also regulates lymphangiogenesis in vivo. Although a role for VEGF-A in wound healing, tumour metastasis and tumour-associated lymphangiogenesis has been demonstrated, a reduced effect was observed in immunocompromised mice (63,66). VEGF-A can also influence lymphatic vascular growth indirectly by recruiting VEGF-C/D-producing macrophages (69).

Angiopoietin-TIE signalling in vascular regulation

Besides the VEGF receptor system, the TIE tyrosine kinase receptors and their angiopoietin ligands are recognised as the second vascular-specific signalling system, essential during embryonic vessel maturation, establishment of venous identity and adult vascular homeostasis (73,74). The TIE2 signalling balance relies on two key ligands – Angiopoietin-1 (ANGPT1) and Angiopoietin-2 (ANGPT2). ANGPT1 acts as a TIE2 agonist, supporting EC survival and vessel stability (73,74). ANGPT1 can be produced by various cell types, including peri-endothelial mural cells (smooth muscle cells and pericytes), fibroblasts, immune cells (such as neutrophils) and others (73,75). Once produced, ANGPT1 activates TIE2 downstream signalling through Phosphoinositide 3-kinase (PI3K) and serine kinase AKT, leading to the inhibition of the transcription factor Forkhead box protein O1 (FOXO1) and repression of *Angpt2*. In murine skin, ANGPT1 induces blood vessel enlargement, specifically affecting the veins, by promoting proliferation in the absence of angiogenic sprouting during a specific vascular plasticity window in the neonatal development (76). Notably, systemic overexpression of ANGPT1 during chronic airway inflammation causes the remodelling of capillaries to post-capillary venules, expressing markers for leukocyte trafficking, such as ICAM1 and P-selectin (75). ANPGT1 has TIE2 agonist functions also in LEC, promoting lymphatic proliferation and sprouting (77).

Contrary to ANGPT1, ANGTP2 is primarily produced by the ECs and generally functions as a TIE2 antagonist in the BECs. ANGPT2 levels increase in response to hypoxia, VEGF stimulation or inflammatory cytokines such as tumour necrosis factor (TNF) during acute inflammation and cancer. This leads to reduced ANGPT1-TIE2 signalling, increased FOXO1 activity and ANGPT2 upregulation, leading to destabilisation of endothelial monolayers (Augustin et al., 2009; Saharinen et al., 2017). In contrast to BECs, in the lymphatic vasculature, ANPGT2 acts as a TIE2 agonist, promoting lymphangiogenesis (78). Genetic deletion of *Angpt2* in LECs or inhibition of ANGPT2 using blocking antibodies leads to decreased VEGFR3 on LECs and reduced lymphangiogenesis. Notably, VEGF-C-induced ANGPT2 production in LECs is involved in the activation of the downstream PI3K-AKT-mTOR pathway (79).

Vascular malformations

Hallmarks and genetics of vascular malformations

Vascular anomalies contain a spectrum of conditions that can manifest as insignificant birthmarks to critical life-threatening disorders. They are broadly classified into proliferative vascular tumours and benign vascular malformations (80). Vascular anomalies develop due to germline or somatic genetic mutations in ECs. Contrary to inherited mutations, somatic mutations are confined to a specific cell type and specific localised area of the body, leading to the manifestation of the mutated gene in the form of genetic mosaicism (81). Vascular malformations are considered monogenic disorders. For instance, a somatic activating mutation in the *PIK3CA* gene, which encodes the catalytic subunit p110α of the PI3K enzyme, appears sufficient for the development of vascular malformations. Intriguingly, the same activating mutations are also found in a group of rare disorders, causing overgrowth of parts of the body, called *PIK3CA*-overgrowth spectrum (PROS), as well as in epithelial cancer (**Figure 5**). However, cancer development is a multi-step process that requires additional somatic mutations beyond *PIK3CA* (82,83). A cancer-like mechanism was recently described in a severe form of Cerebral Cavernous Malformations (CCM). In this case, aggressive vascular malformations form after a three-hit mechanism of consecutive somatic mutations or a combination of a germ line and two somatic mutations. Two of the mutations lead to the loss of function of vascular suppressors (*CCM* genes), while the third mutation acts as a vascular activator (*PIK3CA* gene) (84).

The majority of vascular anomalies are localised vascular malformations associated with activating somatic mutations detected in genes essential for vascular development, proliferation, and apoptosis (81). Most localised lesions are congenital or present right after birth and grow proportionally with the patient's body (83). Exceptions occur when infection or trauma causes rapid lesion growth, necessitating immediate intervention (85). Vascular malformations can lead to pain, thrombosis, tissue destruction and organ dysfunction, which in severe cases can be lethal (83). They are categorised based on clinical features, depending on the affected vessel type and their flow rate, as low-flow capillary malformations, venous malformations (VM) and lymphatic malformations (LM), as well as high-flow arteriovenous malformations (81). Patients can develop mixed or combined vascular lesions, such as lympho-venous malformations. Intriguingly, it appears that fast-flow vascular lesions primarily develop due to mutations affecting the MAPK-ERK pathway or TGF-β-signalling, whereas mutations in the PI3K-AKT pathway cause the majority of slow-flow VMs and LMs (86). Studies show that 60% of the sporadic VMs are caused by somatic activating mutations in the *TEK* gene encoding TIE2 (86,87). This leads to downstream activation of the PI3K-AKT-mTOR signalling, driving the formation of vascular lesions. Additionally,

somatic activating mutations in the *PIK3CA* gene cause 25% of VMs (26,83,87) and the majority of LMs (88,89).

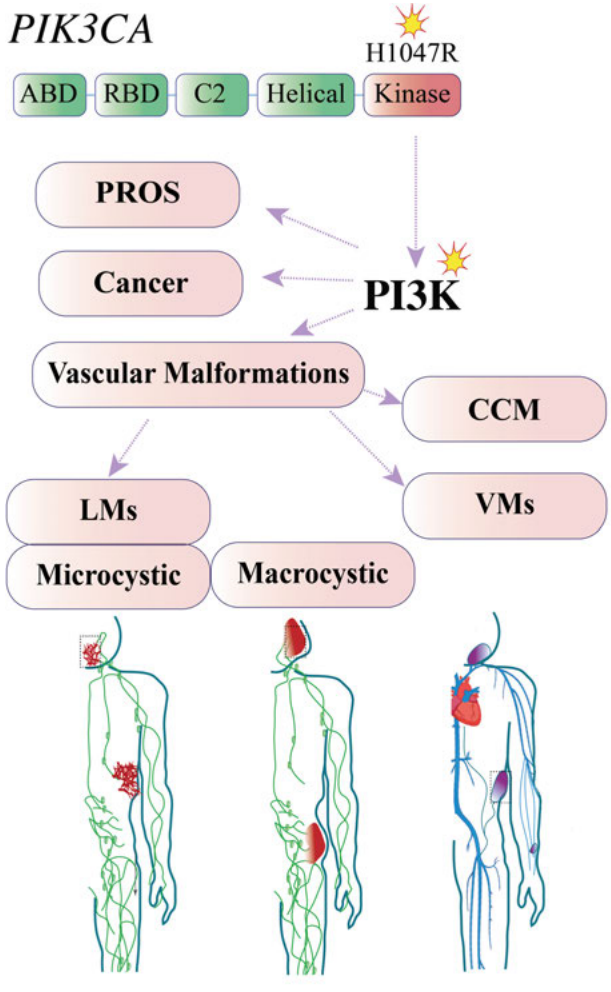


Figure 5. Pathologies caused by somatic activating mutations in *PIK3CA*. Somatic activating mutation H1047R and other mutations in *PIK3CA* cause *PIK3CA*-overgrowth spectrum (PROS), epithelial cancer, and vascular malformations affecting lymphatic vessels or veins. Lymphatic malformations (LMs) are classified as microcystic or macrocystic LM, depending on their morphological features (presented in red in the figure). Venous malformations (VMs) are shown in purple. Recently, *PIK3CA* mutations have been found to be involved in the formation of aggressive Cerebral Cavernous Malformations (CCM).

PI3K signalling and venous malformations

PI3K enzyme is composed of three catalytic subunits (p110 or PI3Kα, β, δ) and five regulatory (p85) subunits, with the latter serving to inhibit kinase activity under normal conditions in the absence of a ligand. The essential role of PI3Kα in the regulation of vascular development and growth, including EC migration, survival, and proliferation, is well established (90). Genetic loss-of-function studies in mice have revealed its importance during normal blood and lymphatic vessel development (46,91,92). The activation of the PI3Kα is initiated when a growth factor, for instance, ANGPT, VEGF-A, or VEGF-C, binds to its receptor tyrosine kinases such as TIE2, VEGFR2 or VEGFR3, leading to their phosphorylation. The activation of the receptor tyrosine kinase leads to the recruitment and binding of p85 to its phosphorylated tyrosine residues, releasing PI3Kα inhibition. Activated PI3K subsequently phosphorylates phosphatidylinositol-4,5-biphosphate to phosphatidylinositol-3,4,5-triphosphate that acts as a docking site for the recruitment of AKT to the plasma membrane where it is activated through phosphorylation. Key downstream targets of AKT include mTOR and FOXO1, responsible for the regulation of essential processes such as cell proliferation, migration, apoptosis, and survival (93). Most activating *PIK3CA* mutations in vascular malformations and cancer are localised to three hotspot locations, namely the helical (E542, E545) and kinase (H1047R) domains of the PI3K enzyme (94). These mutations activate p110α oncogenic potential by increasing the lipid-binding and basal activity of the enzyme using distinct mechanisms (95). The natural transition from inactive to active cytosolic enzyme conformation has four dynamic conformational events, which can combine its effect and synergistically enhance the PI3K activity in cancer cells (95,96). Considering that a distinct mutation activates PI3K to various degrees, it is challenging to establish a clear correlation between PI3K activation and a specific cellular phenotype. However, a recent study on human tissues suggests that the level of mosaicism reflects the potential strength of the pathogenicity of the mutation. For instance, non-hotspot *PIK3CA* mutations are more frequently found in overgrowth syndromes with a broader mosaicism, suggesting a weaker effect compared to LMs, which are due to a hotspot mutation (97).

PIK3CA-driven lymphatic malformations

PIK3CA-driven LMs belong to a broader group of lymphatic anomalies whose classification is complex and mainly based on clinical manifestations. These anomalies include conditions arising from germ-line mutations, such as lymphedema, or somatic mutations, which can manifest as localised solitary lesions (LMs) or in a multifocal manner (complex lymphatic anomalies). LMs, the main focus of this thesis, are localised lesions with an estimated overall prevalence of approximately 1:4000 live births (12). Similar to other vascular

malformations, LMs are typically diagnosed in the first two years of age, but in rare cases, they may become evident during adulthood. Depending on the size of the cysts, LMs can classified as macrocystic or microcystic. Macrocystic LM are characterised by fluid-filled cysts larger than 2 cm in diameter, while numerous multi-lesional microcystic LMs and mixed LMs are smaller than 2 cm in diameter (Ghaffarpour et al., 2022). Among them, microcystic LMs are often diffuse and infiltrate the tissue, leading to more severe and challenging interventions for the patient (12). A recent study indicates that more severe microcystic LMs are associated with a higher frequency of hotspot mutations. In contrast, macrocystic LMs are more commonly identified with non-hotspot *PIK3CA* mutations (98).

Approximately 70% of the LMs manifest in the head and neck region, leading to airway compression and breathing difficulties (85). Additionally, LMs have been detected in the abdominal and thoracic regions, which can cause obstruction of vital internal organs, resulting in life-threatening conditions. In some cases, spontaneous or traumatic intralesional bleeding can occur (Ghaffarpour et al., 2022). LMs are highly reactive to inflammation and infection. Consequently, trauma or bleeding can lead to their rapid growth and complications. Notably, the lumen of the LMs in patients has been found to be filled with cytokines, macrophages, and lymphocytes (Ghaffarpour et al., 2022). An increase in macrophages has also been observed in skin sections of patients with LMs compared to those with healthy skin (99). In addition, lymphoid follicles were reported in the cyst walls of patients with macrocystic LM as early as 1913 (100). More recent immunochemistry studies have shown that lymphoid infiltrates in patients with LMs attract different types of immune cells, such as macrophages, dendritic cells, and B and T lymphocytes (99). These infiltrates also exhibit markers typically found in tertiary lymphoid organs (TLOs), including the critical chemokines CCL21 and CXCL13 (101). TLOs are known as well-organized cell aggregates of lymphocytes and antigen-presenting cells, structured as a primary lymph node-like formation, and associated with adverse effects during chronic inflammation (102). Interestingly, a correlation is found between the formation of TLOs and inflammation-induced lymphangiogenesis within LMs, whereas the centre of TLOs is enriched with colony-stimulating factor, $CD68^+$ macrophages and VEGF-C (99).

Treatments for vascular malformations

Previously, the only options for the treatment of vascular malformations have been invasive methods such as surgical intervention, embolisation, laser ablation and sclerotherapy. In cases of extensive infiltrative lesions, implementing certain options may be impossible or lead to non-curative results (103). Recently anticancer drugs are actively repurposed for trials targeting vascular malformaitons (81). The most frequently used drug rapamycin (sirolimus),

which targets the PI3K downstream effector mTOR, is currently part of several clinical trials (27,104–106). Preliminary results from the latest clinical trials have identified rapamycin as highly efficient in treating slow-flow vascular malformations in children and adults (105). Although rapamycin has shown promise in improving the quality of life for patients, it is important to note that it does not represent a cure, since the treatment rarely leads to the regression of the malformation (104). Rapamycin induces cell cycle arrest, and it is therefore highly effective for inhibiting actively proliferating and growing tissues, but it may be less effective in inducing regression after tissue growth (12,107). Rapamycin is also an immunosuppressive agent, primarily affecting macrophages and T-cells (108–110). This suggests that rapamycin may additionally target paracrine signalling during vascular malformation growth, which may be of importance in inflamed LMs discussed above. Although EC-autonomous mutations are the main driver of vascular malformations, understanding the involvement of immune cells or other paracrine mechanisms during the pathogenesis of vascular malformations could lead to new and effective treatment options.

Interaction between lymphatic endothelium and immune cells

Immune cell trafficking through the lymphatic vessels

The peripheral lymphatic vessels connect the adjacent tissues to the draining secondary lymphoid organs, including the lymph nodes, where adaptive immune responses are initiated. Serving as the main conduit for immune cells, lymphatic vessels allow for close interactions between the LECs and various leukocytes, aiding numerous physiological processes, including immune cell migration.

In the skin, macrophages, mast cells, dendritic cells (DCs), T lymphocytes, and rare innate lymphoid cells are present during homeostasis. However, the main cell types that migrate through the lymphatic vessels at this stage are the antigen-presenting DCs and antigen-experienced T cells. While the transport of DCs is essential for triggering protective immunity and immune tolerance in the lymph node, T cell trafficking is vital for regulating the immune response in the peripheral tissues. Low-level immune trafficking through the lymphatic vessels during the steady state increases rapidly during inflammation. The composition of the skin microenvironment also changes, with monocytes, neutrophils and memory T cells recruited to the site of inflammation (111).

The process of leukocyte trafficking occurs in a series of steps. Initially, immune cells move from the interstitial space towards the lymphatic vessels. Subsequently, they actively enter the vessel, crawl inside, detach themselves and ultimately get passively transported with the lymph flow to either the inflamed tissue or the draining lymph node. The main route of migration for DCs and T cells through the interstitial extracellular matrix towards the lymphatic capillaries is guided by the LEC-expressed C-C motif ligand 21 (CCL21) flow-induced chemotactic gradient. CCL21 also regulates the docking, crawling, and transmigration of DCs from the lymphatic capillaries to the collecting lymphatic vessels (111,112). During inflammation, TNF-α triggers additional release of CCL21 from LECs, leading to an increased influx of DCs expressing the CCL21 receptor CCR7 towards the lymph nodes (113). Then, CCL21 promotes the trans-lymphatic endothelial migration of DCs through a β2 integrin-dependent mechanism (113). The additional interaction between DCs and capillary LECs triggers a calcium-dependent release of CCL21, promoting further DC transmigration (114). Once immune cells reach the collecting lymphatics, the lymph flow rate increases rapidly, leading to the passive transport of leukocytes to the adjacent lymph node (111). Contrary to the previous belief that immune cells enter only through lymphatic capillaries, a recent study has shown that during inflammation, DCs can use CCR7- and β1-dependent mechanisms to enter the collecting vessels for rapid transportation to the lymph node. This entry is mediated by the upregulation of adhesion

molecules on the lymphatic collectors, such as ICAM-1 and VCAM-1 (115). Notably, CCL21 can promote the trafficking not only of DCs but also of subsets of CCR7-expressing T cells, macrophages and neutrophils (111,116,117).

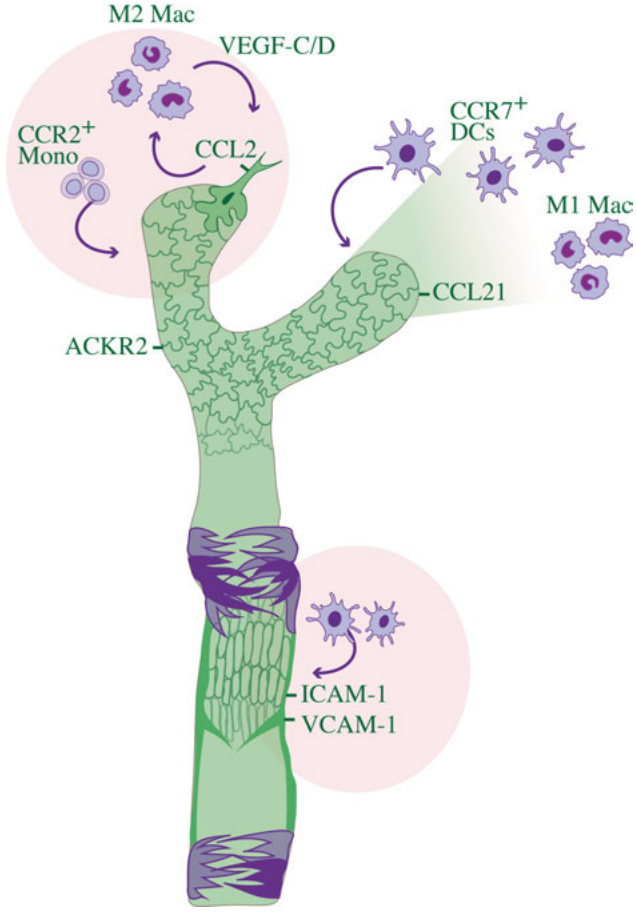


Figure 6. Interaction of peripheral lymphatic vessels with immune cells during homeostasis and inflammation. Capillary LECs guide the trafficking of CCR7$^+$ dendritic cells (DCs) and M1-type macrophages through CCL21 gradient both in homeostasis and inflammation. Additional rapid trafficking of DCs facilitated by the expression of ICAM-1$^+$ and VCAM-1$^+$ on lymphatic collectors occurs during inflammation. During inflammation, capillary LECs upregulate expression of CCL2, which is the main chemoattractant of CCR2$^+$ monocytes and pro-lymphangiogenic M2-type macrophages. The decoy receptor for inflammatory cytokines ACKR2 regulates immune cell trafficking by scavenging extracellular CCL2.

Monocyte recruitment and CCL2-CCR2 signalling

Besides DCs, the monocyte can be directly recruited to the infected tissue and can also enter the lymph node through the afferent lymphatics. Depending on the expression of unique surface molecules and chemokine receptors, murine monocytes are classified into two distinct functional types: classical and non-classical monocytes (118). The non-classical monocytes express high levels of CX_3CR1 and low levels of CCR2 and LY6C. These monocytes are often found adherent or migrating along small blood vessels in a process called patrolling and are important for EC integrity (118). In some cases, they can be recruited to the site of inflammation and play a role in wound healing and tissue repair (119).

In contrast, classical monocytes, also called inflammatory monocytes, are characterised by the high expression of the markers LY6C, CD11b and CC-chemokine receptor 2 (CCR2) and low levels of CX_3CR1. In uninfected mice, their percentage is around 2-5% of all leukocytes and increases rapidly during inflammation or infection with diverse pathogens (119). Once they arrive at the site of inflammation, the classical monocytes can differentiate into different types of DCs or inflammatory macrophages. During dermal infection, monocytes can carry microbial antigens to local lymph nodes, where they can either transfer the antigens to classical DCs or differentiate into monocyte-derived DCs, supporting pathogen-specific T-cell immune responses (119).

During homeostasis, the classical monocytes remain in circulation for a day before they replenish tissue-resident macrophages in the dermis, intestine, pancreas, heart, and lung or differentiate into non-classical monocytes (118). In depletion experiments of resident macrophages, classical $LY6C^{high}$ monocytes can give rise to various tissue-specific macrophages. However, these experiments do not always reflect the mechanism in natural conditions (119). For example, Langerhans cells are maintained by local self-renewal during homeostasis but through a CCR2-dependent monocyte-recruitment mechanism in inflamed skin (120). However, the capacity of monocytes to reconstitute distinct tissue-resident macrophages by adapting their transcriptional profile to share similarities to the specific resident subtypes suggests an essential physiological role in inflammation and infection (118,119). Interestingly, in a steady state, $LY6C^{high}$ monocytes can migrate to the tissue and then remain in their monocyte-like state without further differentiation, acting as a local monocyte reservoir (118,121).

Notably, the high expression of the CCR2 receptor on $LY6C^{high}$ proinflammatory monocytes is crucial for their recruitment during inflammation. CCR2 has two ligands, CC-chemokine ligand 2 (CCL2, called monocyte chemoattractant 1 (MCP-1)) and CCL7 (also known as MCP-3), known for their role in recruiting $LY6C^{high}$ proinflammatory monocytes. During homeostasis, the egress of monocytes from the bone marrow to the circulation is dependent on CCR2-CCL2 signalling, and the number of $LY6C^{high}$ monocytes is

dramatically reduced in CCR2-deficient mice (122). Genetic deletion of *Ccl2* or *Ccl7* during infection reduces monocyte recruitment by 40 to 50% (119). CCL2 is considered to regulate cell migration and disease progression by influencing the activity of the NF-kB, AKT and ERK signalling pathways in various pathologies, such as neuroinflammatory diseases, rheumatoid arthritis, cardiovascular diseases, cancer and metastasis. During malignancy, both cancer and stromal cells can express CCL2 and recruit macrophages (123). A low baseline expression of CCL2 in LEC has been detected during homeostasis (124,125). Notably, CCL2 is found to be upregulated by inflamed LEC of the lymphatic capillaries, suggesting their potential role in monocyte recruitment (124).

Intriguingly, different chemokines attract distinct types of macrophages. CCL21-CCR7 signalling recruits proinflammatory so-called M1-type macrophages, while CCL2-CCR2 primarily attracts anti-inflammatory M2 alternative macrophages, known for their roles in fibrosis, wound healing, and angio- and lymphangiogenesis, discussed in the next chapter (116).

Macrophage phenotypic continuum

Macrophages are the first line of defence of the immune system, specialised in clearing the interstitial environment by detecting, engulfing and eliminating cellular debris or apoptotic and transformed cells, as well as any foreign pathogens and materials. Macrophages can also present antigens to the T cells or produce cytokines essential for immune cell recruitment and regulation of the immune response. They are a heterogeneous and organ-specific immune cell population with exceptional plasticity that allowing for phenotypic and functional shifts that can lead to disease progression or resolution (126,127). Macrophages are described as two main types, M1 and M2, identified by their function and expression. M1-type macrophages are usually activated by interferon-gamma (INF-y) or lipopolysaccharide (LPS) and secrete pro-inflammatory molecules such as IL-1, IL-12, IL-18 and TNF. They can be recognised by high expression of MHC class II and CD68 and their function to eliminate pathogens by driving T cell response (126,127). In contrast, M2-type macrophages, often expressing CD163, can be activated by IL-4 or macrophage colony-stimulating factor (CSF1) to produce anti-inflammatory cytokines such as IL-10. Consequently, their role is connected with extracellular matrix remodelling, tissue repair, angiogenesis, lymphangiogenesis and resolution of inflammation. In reality, macrophages rarely exist as a well-defined M1 or M2 type but within a phenotypic continuum, which can change during disease progression. Insufficient phenotypic transition between the two types may contribute to complications during pathologies such as atherosclerosis, chronic infections, and cancer (127).

Challenges in defining macrophage subtypes come not only from their extreme plasticity but also from their high heterogeneity between the tissues and

even within a single tissue during homeostasis and pathology (128). For instance, within the skin, macrophages can take the identities of either dermal macrophages or Langerhans cells, depending on their location and specialised functions. The Langerhans cells are unique antigen-presenting immune cells serving as essential gatekeepers of the barrier immunity in the epidermis. They can eliminate invading pathogens and present antigens to T cells, thus aiding the control of epidermal tolerance. On the other hand, a subset of dermal macrophages functions as vessel-associated macrophages, important for blood vessel integrity, fibrosis and immune cell recruitment, or as sensory nerve-associated macrophages, essential for nerve regeneration (128).

The resident macrophages in the fluid of the peritoneal cavity are well characterised, with two distinct types identified based on their development and function – $CD11b^{+}F4/80^{+}$ GATA6-dependent large peritoneal macrophages and $MHCII^{+}$ IRF4-dependent small peritoneal macrophages (129,130). In contrast, macrophages associated with the mesenteric membrane have not been as extensively studied. A recent study described them as $LYVE1^{hi}$ $MHCII^{lo}$ and $LYVE1^{lo}$ $MHCII^{hi}$ subsets, similar to the interstitial macrophages found in other organs such as the lung (130,131). $LYVE1^{lo}MHCII^{hi}$ macrophages display an exceptional antigen-presenting capacity, which can promote $CD4^{+}$ T cell proliferation and differentiation, indicating their immune-regulatory potential. On the other hand, the $LYVE1^{hi}MHCII^{lo}$ subtype plays a crucial anti-fibrotic role by repressing immune cell infiltration, inflammation and collagen deposition in heart and lung fibrosis models. Their depletion correlates with the upregulation of innate immune cells in the lungs of bleomycin-treated mice, suggesting that their absence during inflammation promotes an influx of monocytes, DCs and neutrophils (131).

Pro-lymphangiogenic macrophages in cancer and inflammation

Within the diverse macrophage populations in various tissues, specifically during malignancy and inflammation, macrophages that express F4/80, CD11b, CD68, and LYVE1 are often identified as pro-lymphangiogenic by their high expression of growth factors such as VEGF-C and VEGF-D (132). For instance, human tumour-associated macrophages (TAMs) of squamous skin and uterine cervix carcinomas are found to be a significant source of VEGF-C/D, causing peritumoral lymphangiogenesis (133,134). Macrophages also trigger VEGF-C/VEGFR3-dependent lymphatic growth in chemotherapy-treated tumours, promoting the metastatic process (67). Intriguingly, tumours with high expression of TNF-α are associated with high levels of both lymphangiogenesis and angiogenesis. Replacement of wild-type TAMs with TNF-α-receptor TNFR1-deficient TAMs leads to their inability to be recruited after TNF-α-stimulation. Consequently, the lack of accumulated TAMs inhibits lymphangiogenesis and cancer cell metastasis to the lymph node without

affecting angiogenesis. This indicates a crucial role of TNF-recruited VEGF-C producing macrophages for tumour lymphangiogenesis (135). Another evidence supporting the importance of macrophages during cancer lymphangiogenesis is that their depletion leads to the reduction of lymphatic vessels in various tumour types (127,136). In addition to producing VEGF-C, TAMs can also produce VEGF-A, thus influencing lymphangiogenesis indirectly by promoting VEGF-A-VEGFR1-mediated recruitment of pro-lymphangiogenic macrophages during cancer and inflammation (136,137). In some situations, macrophage-derived VEGF-A may directly affect LECs through VEGFR2-signaling, as shown in vitro and during wound healing (70–72).

Another mechanism through which macrophages can influence lymphatic vessels is by producing metalloproteases and triggering ECM remodelling. Intriguingly, Podoplanin (PDPN), which is used widely as a marker for lymphatic vessels, is also expressed in specific types of TAMs in breast cancer, recruited around the lymphatic sprouts. They both promote ECM remodelling by regulating MMPs and collagens and enhance lymphangiogenesis and metastasis (138). Some studies have also suggested that macrophages can incorporate or transdifferentiate into LECs (139,140), while others only observe them adjacent to lymphatic vessels (138). Notably, evidence for macrophage transdifferentiation comes predominantly from experiments done in vitro or in hyperinflammatory models, far from physiological conditions (136).

Besides cancer, a high accumulation of macrophages has been observed in skin biopsies of patients with breast cancer-related lymphedema as well in a mouse tail model of lymphedema (141,142). In the latter, the infiltrated macrophages are identified as $CD11b^{+}F4/80^{+}$ M2-type and serve as a significant source of VEGF-C, contributing to the initiation of pathology by triggering excessive immature lymphatic vessel formation (142). Clodronate- or diphtheria toxin-induced depletion of macrophages led to a 60 to 70% downregulation in their numbers, followed by a significant reduction in *Vegfc* and suppression of edema. The same study showed that the expression of humoral factors such as INF-γ and IL-17 in $CD4^{+}$ T cells enhanced VEGF-C expression in the macrophages. This promoted the generation of new immature lymphatic vessels, contributing to the accumulation of interstitial fluid, at least partially by activating VEGF-C production in macrophages (143). Interestingly, the interaction between LECs, pro-lymphangiogenic macrophages and $CD4^{+}$ T cells is thus essential for both the initial lymphangiogenesis during the primary stage of acute edema and long-term chronic lymphedema pathology (142,143).

Pro-lymphangiogenic macrophages are also essential in other inflammation-related diseases. For instance, $CD11b^{+}$ macrophages produce VEGF-C to trigger LPS-induced lymphatic growth and remodelling in the diaphragm of mice with peritonitis (144) and in the skin of mice with atopic dermatitis (145). Inflammation-induced lymphangiogenesis in the inflamed, usually avascular cornea is also triggered by VEGF-C-expressing $CD11b^{+}$ macrophages, and

local macrophage depletion leads to its inhibition (137,146). The depletion of $CD11b^+$ macrophages during bacterial pathogen-induced acute inflammation in the skin leads not only to the blocking of VEGF-C/D or -A signaling but also to the reduction of inflammatory cell migration, antigen clearance, lymph flow and inflammation resolution via the draining lymph node (68).

Accumulation of M1- and M2-type of macrophages is found in the skin of patients with LMs. Intriguingly, in infected LMs, the predominant population was identified as $CD68^+CD163^+$ M2-type macrophages, suggesting a pro-lymphangiogenic function. This was supported by the correlation between the presence of CD68 macrophages and VEGF-C expression in these LMs (99).

In addition to macrophages, other immune cells of both innate and adaptive immunity have been recognised as active regulators of lymphatic growth and function by producing cytokines and growth factors (147). For instance, mast cells, DCs and neutrophils can promote lymphangiogenesis by producing VEGF-C and/or VEGF-D and VEGF-A during inflammation and cancer (148–150). Lymphocytes in the lymph node can regulate lymphangiogenesis as well. While B-cells promote lymphatic expansion in an inflamed lymph node, INF-γ-producing T cells can inhibit lymphangiogenesis (151,152). Notably, T cells can negatively regulate lymphatic function in numerous diseases including lymphedema, asthma and inflammation directly by producing anti-lymphangiogenic cytokines or indirectly by recruiting pro-inflammatory macrophages (147,153–155).

LECs as active regulators of the immune response

Previously, lymphatic vessels were viewed as a passive route for leukocytes and antigen trafficking. However, they have recently been recognised as active participants in regulating various physiological responses. For instance, LECs of the lymph node can modulate different aspects of immunity by regulating immune cell migration and recruitment through the production of cytokines and pro-inflammatory molecules that directly affect the immune cells. A recent study has shown that specific lymph node LECs are crucial for producing CSF1- the major growth factor for sinusoidal macrophages (156). This suggests that LECs create a favourable microenvironment for the development and differentiation of macrophages in the lymph node. Additionally, lymph node LECs actively mediate peripheral immune tolerance by presenting and achieving antigens (2,157).

Notably, a new subtype of lymph node LECs localised in the medullar sinus, called Ptx3-LECs, was recently found through single-cell RNA sequencing. These cells express genes involved in lymphangiogenesis and lymphocyte egress and regulation, including *Ptx3*, *Lyve1, Ccl21*, *Mrc1, Itih5,* and *Ackr2* (8). *Ptx3,* also known as TNF-stimulated gene 14 (TSG-14), belongs to the pentraxin family and is involved in various pathophysiological conditions, including inflammation, tumour expansion and invasion, tissue repair,

cardiovascular diseases, asthma, rheumatoid arthritis, kidney diseases, sepsis and COVID-19 (158). PTX3 binds pathogens or damaged self-proteins as a soluble pattern recognition molecule (159). For instance, during inflammation, PTX3 can activate the complement system and, by regulating both pro- and anti-inflammatory cytokines, can promote immune cell infiltration and polarisation (158). Additionally, PTX3 binds to collagen and other ECM proteins, suggesting a role in matrix remodelling and tissue repair (159).

In cardiovascular diseases, PTX3 is involved not only in cytokine regulation but also in direct or indirect modulation of nitric oxide levels, leading to endothelial dysfunction. Besides regulating oxidative stress, PTX3 can form a complex with FGF2, inhibiting angiogenesis. However, its effects on the endothelium and angiogenesis remain controversial, similar to its pro- and antitumor effects in different malignancies (158,160). While PTX3 reduces the proliferation and metastasis in some cancer types, it promotes tumour growth in others and its high expression is typically associated with a poor prognosis (161,162). The role of PTX3 in the peripheral lymphatic vasculature is not well studied. A recent study showed inhibition of VEGF-A/FGF2/Sphingosine-1-phosphate-induced lymphangiogenesis after treatment with recombinant human PTX3 in vitro and in PTX3-overexpressing transgenic mice in vivo. This suggests that PTX3 may act as a negative modulator of lymphatic growth, potentially due to its ability to modulate the activity of FGF and other growth factors (161).

LECs in the peripheral vasculature have also been shown to play an active role in paracrine signalling, influencing various cell types, such as epithelial cells, cardiomyocytes, and adipocytes, and regulating injury-mediated regeneration (163,164) and adipose thermogenesis (165). During malignancy, peripheral LECs acquire functions similar to those of lymph node LECs, contributing to an immunosuppressive tumour microenvironment. An example is the elevated PD-L1 expression on LECs in tumour-draining lymph nodes, which has also been observed in dermal LECs in response to IFNγ in melanoma, leading to the inhibition of cytotoxic T cell accumulation (166,167). Peripheral dermal LECs also play a role in antitumor immunity by acting as antigen-presenting cells expressing MHCII and MHCII-related antigen-presentation machinery (168). A similar immunoregulatory role of LECs through antigen presentation has been recently observed beyond the tumour microenvironment, specifically in meningeal lymphatic vessels during neuroinflammation (169).

Aim

The aim of this PhD thesis was to increase our understanding of the endothelial cell-autonomous and paracrine mechanisms underlying pathological vascular growth associated with *Pik3ca*-driven lymphatic and venous malformations, with the ultimate goal of proposing a rationale for more effective therapeutic strategies for patients with these diseases.

Present Investigations

Paper I

Blockade of VEGF-C signalling inhibits lymphatic malformations driven by oncogenic *PIK3CA* mutation, Nat Commun, 2020

Somatic activating mutations in the *PIK3CA* gene lead to overactivation of PI3K enzyme and the downstream AKT-mTOR signalling, causing the majority of LMs (88,89). LMs manifest as either large cysts, predominantly in the neck region, called macrocystic LMs, or as tissue-infiltrative microcystic LMs that are challenging to treat (12).

In this study, we aimed to establish a mouse model of *Pik3ca*-driven LM that recapitulates the key features of human disease and use it to characterise the mechanisms underlying pathological lymphatic vessel growth. For this purpose, we focused on one of the most common activating *Pik3ca* mutations, H1047R, a cause of both macrocystic and microcystic LMs in humans. Using a mouse model of inducible $Pik3ca^{H1047R}$ expression in LECs, we observed the formation of lesions that closely resembled those observed in patients. Lesion morphology was dependent on the development timing of PI3K activation. Embryonic induction of $Pik3ca^{H1047R}$ expression led to large lymphatic cysts in the neck region, reminiscent of macrocystic LM. In contrast postnatal activation of PI3K signalling induced extensive lymphatic vessel sprouting and promoted progressive growth of lesions resembling microcystic LM.

Studies in primary mouse LECs in vitro revealed increased cell migration and high basal activity of the downstream effector AKT in $Pik3ca^{H1047R}$ expressing LECs. Stimulation with VEGF-C in vitro caused a further increase in AKT activity beyond that observed in control LECs, suggesting that $Pik3ca^{H1047R}$ expressing LECs are exquisitely sensitive to VEGF-C. The involvement of VEGF-C signalling in LM pathogenesis in vivo was supported by increased levels of its receptors, VEGFR3 and NRP2, in newly formed lymphatic sprouts, as well as infiltration of immune cells, including macrophages. Blockade of VEGF-C signalling by using a soluble VEGF-C trap led to inhibition of LM growth, indicating the dependence of $Pik3ca^{H1047R}$-driven pathological vessel growth on upstream VEGF-C/VEGFR3 signalling. Importantly, a combination treatment with the soluble VEGF-C trap and the mTOR inhibitor Rapamycin led to the regression of abnormal lymphatic vessel sprouts.

In summary, our findings indicate that the co-inhibition of both the upstream VEGF-C/VEGFR3 and the downstream AKT-mTOR pathway provides an effective strategy for the treatment of *Pik3ca*-driven microcystic LM in mice, presenting a potential avenue for clinical applications in patients.

Paper II

Immune-interacting lymphatic endothelial subtype at capillary terminals drives lymphatic malformation, *JEM*, 2023

While the majority of the LM are caused by somatic activating mutations in *PIK3CA*, around 25% of VM also arise from the same mutations (26,87,170). However, the vessel subtype-specific pathological mechanisms associated with activation of PI3K signalling have not been explored. Consequently, the first aim of this study was to compare the $Pik3ca^{H1047R}$-driven mechanisms underlying VM and LM pathology.

To allow BEC-specific expression of $Pik3ca^{H1047R}$, we first generated a novel $Vegfr1\text{-}CreER^{T2}$ mouse line. We then utilised a robust model of progressive VM and compared it to the microcystic LM model presented in Paper I. Analysis of *Pik3ca*-driven vascular phenotype revealed endothelial-subtype-specific responses. While lymphatic vessels expanded by sprouting, blood capillaries and veins showed localised vessel dilation, with no apparent effects on the arteries.

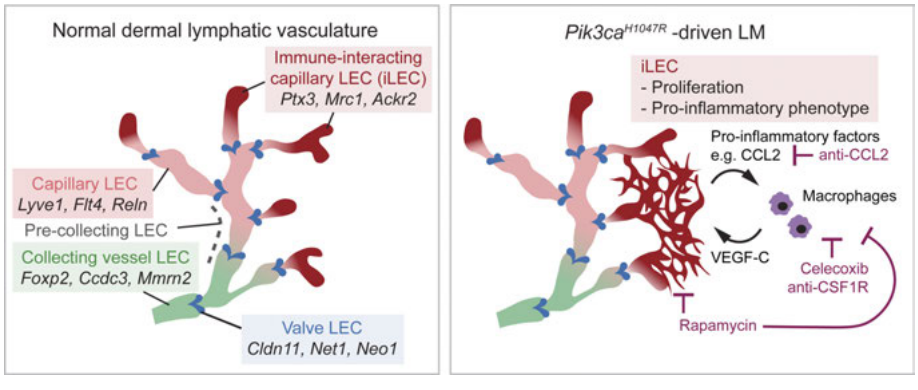
We found that the induction of both lymphatic and blood vascular growth involved extensive proliferation, suggesting an EC-cell-autonomous effect of PI3Kα activation during the initial stages of lesion development. Interestingly, while advanced LM lesions remained proliferative, BEC proliferation returned to baseline in the advanced VM lesions.

Sustained LEC proliferation in the LM model was associated with the infiltration of myeloid immune cells, a phenomenon previously observed both in patients and mice with LMs (99), Paper I). Myeloid cells are known to be a significant source of pro-lymphangiogenic growth factors in various conditions. Given the pro-lymphangiogenic role of immune cells and the heightened LEC sensitivity to VEGF-C in LMs (Paper I), the second aim of this study was to explore the paracrine signalling between immune cells and LECs and its contribution to LM growth.

FACS and qRT-PCR analyses defined the accumulated immune cells during the early stages of active vascular growth in murine LMs as VEGF-C-expressing pro-lymphangiogenic macrophages. The increase in macrophages coincided with an increase in tissue cytokine levels, measured by multiplex ELISA. Remarkably, this process was accompanied by an expansion of a newly identified immune-interacting capillary LEC subtype, termed iLEC,

which we characterised using single-cell transcriptomics. iLECs were identified by the expression of *Ptx3*, previously found in LECs within medullary sinuses of the lymph node (8). We fouther found that upon *Pik3ca*^{H1047R} activation, iLECs proliferated and produced pro-inflammatory genes, such as *Ccl2*, associated with macrophage recruitment. Notably, CCL2 blocking, CSF1R-related macrophage depletion and COX-2 inhibition all reduced *Pik3ca*^{H1047R}-driven lymphangiogenesis, showing the importance of macrophage recruitment and signalling in LM growth.

Taken together, we identified a novel subtype of immune-interacting dermal *Ptx3*$^{+}$ LECs located at the capillary terminals of the peripheral lymphatic vasculature. Our data suggests that *Pik3ca*^{H1047R}-driven LEC-autonomous regulation of pro-lymphangiogenic macrophages and immune response critically contributes to LM growth and reveals paracrine signalling as an essential aspect of LM pathogenesis. Furthermore, this discovery provides new insight into the function of peripheral lymphatic vessels in the skin, highlighting their active role in the recruitment and regulation of the immune response during inflammation.



Graphical abstract

Paper III

Organ-specific mechanisms of *Pik3ca*-driven lymphatic malformation, Manuscript, 2023

Approximately 70% of macrocystic LMs manifest in the neck and head region in patients (171). In Paper I, we observed dermal macrocystic LMs in the neck region of murine embryos following LEC-specific induction of $Pik3ca^{H1047R}$ at early developmental stage, while later induction led to microcystic LMs. Yet, the mechanisms underlying these distinct phenotypes remain unknown.

In this study, we aimed to extend our analyses of *Pik3ca*-driven LEC responses from different developmental time points to different organs, with a particular focus on those within the abdomen. Approximately 10% of all macrocystic LMs are localised in the abdominal and thoracic areas, and although rare, they require quick diagnosis and immediate intervention due to close apposition to essential vital organs (172,173). Often, these LMs are located in the mesentery, connected to the small intestine (172,174). Interestingly, we found that, contrary to the skin, postnatal induction of $Pik3ca^{H1047R}$ expression led to the formation of large cystic lesions remiscent of macrocystic LMs in the mesentery. This suggests tissue-specific mechanisms for PI3K-driven pathology.

In summary, we established a new postnatal model of *Pik3ca*-driven macrocystic LM, which opens up possibilities for an in-depth investigation of their underlying mechanism and comparison to the microcystic LM type.

Paper IV

Venous-specific autocrine signalling promotes *PIK3CA*-driven vascular malformations, Manuscript, 2023

Venous malformations are the most common form of vascular lesions (175). The majority of VMs are caused by activating mutations in the *TEK* gene, which encodes the TIE2 receptor, which is essential for venous development and growth. In addition, approximately 25% are due to *PIK3CA* mutations (26,87,170).

Here, we aimed to explore the mechanism of VM growth by using the genetic mouse model of $Pik3ca^{H1047R}$-driven VMs described in Paper II, in combination with single-cell transcriptomics. Analysis of BECs from control mice revealed arteriovenous a zonation, similar to that observed in other organs. $Pik3ca^{H1047R}$ activation in BECs promoted their proliferation and venous identity. In addition, genetic tracing of $Pik3ca^{H1047R}$ -expressing BECs revealed their clonal expansion selectively within veins and venous capillaries. Notably, this expansion did not seem dependent on VEGF-A. Comparison of the

gene expression profiles of mutant BECs with controls revealed upregulation of genes involved in the regulation of metabolic processes, consistent with high PI3K signalling, as well as *Angpt2* as an autocrine regulator of TIE2 signalling. Targeting of the upstream TIE2 signalling inhibited VM growth in mice, and may thus provide a therapeutic opportunity for patients with *PIK3CA*-related VMs.

Future Perspectives and Conclusions

Pre-clinical mouse models as tools for exploring vascular malformations

The identification of activating mutations in *PIK3CA* has provided an opportunity to generate mouse models carrying clinical mutations, mimicking human vascular malformations and increasing our knowledge of the underlying pathological mechanism. Ubiquitous or endothelial-specific expression of *Pik3ca*^{H1047R} during murine embryonic development leads to severe vascular phenotype and lethality (27,176). Thus, using the Cre/loxP system for tamoxifen-inducible and EC-specific activation (177) is necessary to allow for spatially and temporally controlled expression of the mutation. This better mimics the somatic mosaicism observed in the disease and enables an extended period of observation to understand its effects.

Over the last decade, several mouse models using Cre/loxP technology have been described as models of human vascular malformations (26,27,178). These models are characterised by severe alterations resulting from a generalised rather than localised phenotypic response due to systemic administration and high dosages of tamoxifen. In some cases, this has been beneficial, for instance, in exploring the effects of generalised lymphatic anomalies in mice upon LEC-specific induction of *Pik3ca*^{H1047R} expression (178). However, due to severe vascular phenotypes, these models generally survive only up to two weeks, depending on the developmental stage of induction, after tamoxifen administration (26,27). This rapid systemic phenotype does not allow for precise follow-up on vascular changes, and it does not accurately represent the mechanism of the solitary LM and VM lesions observed in patients.

To develop a more accurate model for localised LM and VM, we induced mosaic EC-specific expression of the *Pik3ca*^{H1047R} transgene using the *Vegfr3-CreER*^{T2} (Paper I, II) or *Vegfr1-CreER*^{T2} (Paper II, IV) mice, respectively, by administering a low dose of 4OH-Tamoxifen topically to the ear skin. This approach led to a locally restricted, robust, and progressive vascular growth that could be followed for an extended period of up to 10 weeks (Paper II). The key advantage of our mouse models is the extended lifetime with minimal life-threatening effects on the internal organs and accurate representation of the genetic mosaicism mimicking the human disease. However, a limitation of our model is the inability to genetically label *Pik3ca*^{H1047R} -expressing cells to study their behaviour and effects on neighbouring wild-type cells. Consequently, models that combine induction of low-frequency recombination to

induce single isolated vascular lesions with reliable tracking of *Pik3ca*^{H1047R} -expressing cells should be developed to enhance our understanding of paracrine effects. Such an approach could also extend the observation period, which is likely required to recapitulate the immune response and other long-term effects observed in human patients. Furthermore, this would be beneficial for assessing therapeutic effects.

Targeting of paracrine signalling for the treatment of vascular malformations

Alongside EC-autonomous genetic mutations, our studies and those of others indicate that additional simultaneous paracrine signalling is a critical contributor to LM growth (12). In particular, VEGF-C-VEGFR3 signalling appears to be involved in LM development in both mice and human (12,179,180).

Using our mouse model of progressive microcystic LM established in Paper I, we proposed a therapy combining Rapamycin and VEGF-C-trap to simultaneously inhibit the downstream mTOR and the upstream VEGF-C-VEGFR3 signalling. Remarkably, this combination therapy promoted regression of LM during the observation period. Importantly, VEGF-C can be produced in human tissue by various cell types, including arterial ECs, fibroblasts, and immune cells (181). Particularly, macrophages can promote lymphangiogenesis by being a source of VEGF-C during inflammation and malignancy (136,182) as well as in LM, as reported in Paper II. Although we observed no difference in *Vegfc* transcript levels in the total non-immune cell population isolated from mutant and control mice, we did not investigate if another VEGF-C- or VEGF-D-producing cell type also contributes.

Interestingly, during the advanced stages of LM in our model (at 10 weeks, 7 weeks after induction), FACS data indicated an accumulation of B and T cells (Paper II). This is consistent with the development of B and T cell-rich TLO structures observed in patients with LM (99,183). The accumulation of VEGF-C-expressing macrophages in the centre of these TLOs in patients suggests a potential pro-lymphangiogenic role, but further research is necessary to determine the function of TLOs in LM.

While likely not constituting a standalone cure, targeting the paracrine signalling between immune cells and LECs holds potential benefits for LM patients, especially in cases of immune-reactive inflamed or infected LMs, where tissue rapidly swells (85). Furthermore, combining anti-inflammatory therapy with targeting the EC-autonomous mechanisms may lead to improvements in patients who do not respond to traditional treatment. For instance, a recent study involving a patient with previous ineffective surgery and sclerotherapy showed that the COX-2 inhibitor Celecoxib reduced the growth of LM lesions (184). Additionally, such combination therapies may allow the use of lower drug dosage, consequently leading to fewer side effects and complications for patients. Notably, a clinical trial investigating VEGF-C/D inhibition to target abnormal blood vessels in Neovascular Age-Related Macular

Degeneration has shown positive results, improving patients' vision (185). This suggests that anti-VEGF-C therapy may be a suitable and safe treatment for certain diseases. A future clinical trial may become feasible to assess the potential of paracrine inhibition using anti-VEGF-C therapy for the treatment of LMs.

Contrary to LM, our analysis of the mouse model of VM suggests that *Pik3ca*^{H1047R}-driven blood vessel growth is not dependent on the upstream VEGF-A signalling and is not associated with macrophage recruitment. Further research is needed to explore whether VEGF-A or other immune populations are necessary at a more advanced stage of VM progression. For instance, we detected infiltrating neutrophils and B-cells in advanced VMs, which may be related to the acquisition of post-capillary venous phenotype following *Pik3ca*^{H1047R} expression or to secondary effects such as vessel leakage and tissue hypoxia.

Identification and role of immune-interacting iLECs

The newly discovered dermal immune-interacting LECs, iLECs (Paper II), share both similar transcriptome and morphological features with the Ptx3-LECs in the LN. For instance, they both have blind ends, specialised in attracting leukocytes and fluid into the lymphatics, that can sprout, forming spike-like extensions. The function of the expressed genes defining their specific transcriptome may provide insights into better characterising the role and identity of iLECs.

The protein encoded by the most prominent gene, PTX3, is suggested to function as a negative regulator of lymphatic growth by regulating FGF2. FGF2 promotes LEC proliferation and survival in *in vitro* studies, through PROX1-regulated expression of its receptor FGFR3 (186,187). Our single cell transcriptomics data show that FGFR3 is not, however, expressed in the dermal LECs *in vivo*. FGF2 may also promote lymphangiogenesis indirectly by cooperating with VEGF-C, as reported in inflammatory models such as corneal lymphangiogenesis assay and cancer (187,188). In our study of *Pik3ca*-driven LM, PTX3 was strongly expressed at the tip of abnormal lymphatic sprouts composed of highly proliferating iLECs, suggesting its involvement in lymphangiogenic vessel growth and VEGF-C-VEGFR3 signalling. Furthermore, PTX3 was upregulated in LECs within LM lesions in human patients, further suggesting a role of LEC-produced PTX3 in human pathology. More research is needed to investigate the role of PTX3 in LM pathology and lymphatic vessel growth.

In addition to *Ptx3*, the upregulation genes such as *Mrc1, CD44, L1cam, CD40,* and *Cd276* in the mutant LM-associated iLECs suggest their involvement in the regulation of immune response. The macrophage mannose receptor (MRC1, CD206) regulates immune cell trafficking through the peripheral lymphatic vessels by binding to L-selectin (189). CD44 binds to MRC1 and promotes lymphocyte migration via the afferent lymphatics (190). In addition,

L1cam and *CD40* regulate Langerhans cell trafficking in the skin (191,192), and *Cd276* is important for the recruitment of tumour-associated macrophages (193). However, since these molecules are also known to be essential for adaptive immunity during malignancy (194,195), their precise function in *Pik3ca*-driven LM needs to be further investigated. It is possible that they are involved in regulating the trafficking of T cells, which were found at the advanced stage of our mouse model (Paper II) and in TLOs in LM patients (99).

In *Pik3ca*-driven LM, iLECs were found to produce *Ccl2,* contributing to the recruitment of VEGF-C-producing macrophages and promoting further lymphangiogenesis (Paper II). Interestingly, Ptx3-LECs in the lymph node do not produce *Ccl2* during homeostasis, but its upregulation is observed during oxazolone-induced inflammation (2,8). In contrast, both dermal iLECs and lymph node Ptx3-LECs produce the atypical chemokine receptor ACKR2 in homeostasis. ACKR2 binds to inflammatory chemokines, thereby controlling inflammatory responses. For instance, ACKR2 competes with the CCR2 receptor for binding to CCL2, scavenging it and balancing the pro-lymphangiogenic macrophage recruitment (125,196). Therefore, the function of iLECs may differ between homeostasis and disease. It is possible that in normal conditions iLECs have a passive role and serve as a reservoir for active immunemodulatory iLECs during immune surveillance and pathology. Interestingly, $PTX3^+$ iLECs were recently described in the nasal lymphatic vasculature in both mice and humans (197), as well as in the nasopharyngeal mucosa of adult and aged mice, exhibiting a more substantial proportion than in the lymph node and skin (198). This observation suggests that tissue-specific demands for responding to potential immune challenges may correlate with the abundance of $PTX3^+$ iLECs. Further research is needed to explore the abundance of iLECs in other organs and uncover their roles in homeostasis and pathology.

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