Intravital two-photon microscopy of lymphatic vessel development and function using a transgenic Prox1 promoter-directed mOrange2 reporter mouse

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Abstract
Lymphatic vessels, the second vascular system of higher vertebrates, are indispensable for fluid tissue homeostasis, dietary fat resorption and immune surveillance. Not only are lymphatic vessels formed during fetal development, when the lymphatic endothelium differentiates and separates from blood endothelial cells, but also lymphangiogenesis occurs during adult life under conditions of inflammation, wound healing and tumour formation. Under all of these conditions, haemopoietic cells can exert instructive influences on lymph vessel growth and are essential for the vital separation of blood and lymphatic vessels. LECs (lymphatic endothelial cells) are characterized by expression of a number of unique genes that distinguish them from blood endothelium and can be utilized to drive reporter genes in a lymph endothelial-specific fashion. In the present paper, we describe the Prox1 (prospero homeobox protein 1) promoter-driven expression of the fluorescent protein mOrange2, which allows the specific intravital visualization of lymph vessel growth and behaviour during mouse fetal development and in adult mice.

Structure of the lymphatic vessel system
Higher vertebrates essentially depend on the presence of two vascular networks: the circulatory blood vascular system and the dendriform, unidirectionally functioning, lymphatic vessel system [1]. Lymphatic vessels collect extravasated fluid, macromolecules and cells from the interstitium and return them to the venous arm of the circulation. Besides its important function for tissues fluid maintenance and fat resorption, the lymphatic system is indispensable for immune surveillance and immune response generation. Lymph vessels guide extravasated innate immune cells and soluble antigen to the lymph nodes where both are tested by, and interact with, the cells of the adaptive immunity, most notably B- and T-lymphocytes [2]. Lymphatic vessels are found in almost all tissues, with notable exceptions being avascular tissues, such as epidermis, hair, nails, cartilage and cornea, and some vascularized, but immuneprivileged, tissues that include brain, retina and bone marrow. The lymphoid system also comprises the secondary lymphoid organs: lymph nodes, spleen, thymus, tonsils and Peyer’s patches [3].

Initial lymphatic capillaries are blind-ending thin-walled vessels lined by a single layer of overlapping oak-leaf-shaped endothelial cells that lack pericyte or smooth muscle cell coverage and a continuous basement membrane. In the presence of low tissue fluid pressure, initial lymphatics display little or no lumen. When the interstitial pressure rises, specialized anchoring filaments that connect lymphatic capillaries to the surrounding extracellular matrix exert tension on the endothelial cells, resulting in opening of the lumen and fluid uptake [4]. This process is largely facilitated by the specialized discontinuous cell junctions of lymphatic capillaries that consist of VE-cadherin (vascular endothelial cadherin)-positive buttons, which alternate with VE-cadherin-negative flaps at stretches where the endothelial cells overlap [5]. These interjunctional gaps are also used by leucocytes as sites of vessel entry [6].

The lymph, which consists of interstitial fluid, macromolecules and leucocytes, is drained into pre-collector lymphatic vessels that combine properties of capillaries (oak-leaf-shaped cells) and collecting vessels (intraluminal valves). Transport to the venous circulation is finally accomplished by increasing larger collecting vessels that are lined by a thin continuous basement membrane, show pericyte and smooth muscle cell coverage and consist of smaller functional
units called lymphangions. Each lymphangion is delimited by intraluminal bileaflet valves that ensure directionality of flow and are capable of active lymph transport [1]. The collecting lymphatics drain into the thoracic and right lymphatic duct, which are the only physiological major connections between blood and lymph vessels.

Development of the lymphatic vessel system

Over the last two decades, identification of a number of molecular makers for lymphatic endothelial cells that include the transcription factor Prox1 (prospero homeobox protein 1), the receptor tyrosine kinase or VEGFR3 [VEGF (vascular endothelial growth factor) receptor 3], Lyve1 (lymphatic vessel endothelial hyaluronan receptor 1) and glycoprotein Pdpn (podoplanin) has significantly facilitated research into lymphatic biology [7]. In mice and humans, development of the lymphatic system starts after the primordial blood vascular system has formed [8]. As early as 1902, Sabin [9] postulated that LECs (lymphatic endothelial cells) derive from venous endothelium, a hypothesis that was largely confirmed by the analysis of genetically modified mice over the last decade.

The first molecular marker indicating lymphatic differentiation is Lyve1, which is first expressed in the cardinal vein from E (embryonic day) 9 onwards and indicates lymphatic competence [10]. Then, 1 day later, around E10, polarized expression of Prox1 can be detected, initially in a subset of Lyve1-positive endothelial cells of the cardinal vein [11,12]. Genetic deletion has demonstrated that Prox1 is a master control gene for the determination of LEC fate [13]. Prox1-deficient endothelial cells fail to acquire the LEC phenotype and arrest at E11.5, being incapable of budding and migrating from the cardinal vein [14]. The signals regulating Prox1 expression are not fully understood; however, the homeobox transcription factor Sox18 [SRY (sex-determining region on the Y chromosome)-related HMG (high-mobility group)-box 18], which is also expressed in Lyve1-positive endothelial cells of the cardinal vein before Prox1 expression, appears to act upstream of Prox1 [4,15]. Early during development, VEGFR3 is expressed in all endothelial cells; however, after development of the lymphatic system, expression becomes restricted to LECs. One ligand for VEGFR3 is VEGF-C, which is required for the migration and sprouting of Prox1-positive LECs and for proliferation and survival of LECs until postnatal maturation; VEGF-C-deficient mice die prenatally because they fail to establish a functional lymphatic remodelling and maturation

After separation from the cardinal vein, the emerging lymphatic endothelial cells organize into primary lymph sacs, from which a first lymphatic plexus appears by centrifugal sprouting. Through subsequent maturation, which involves the transmembrane Eph-receptor ligand ephrinB2, this plexus remodels into a functional lymphatic vasculature. Mice with a mutation in the PDZ-domain-binding motif of ephrinB2 fail to remodel the lymphatic vasculature into an organized network of capillaries and collectors and lack luminal valve formation [21,22]. Besides ephrinB2, Ang (angiopoietin) growth factors and their kinase receptors Tie [tyrosine kinase with Ig-like and EGF (epidermal growth factor)-like domains]-1/2 are involved in the remodelling and stabilization of blood and lymph vasculature [23–25].

A further important factor in the maturation of lymphatic vessels is the transcription factor FoxC2 (forkhead box C2), which is expressed in developing lymphatic vessels and adult lymphatic valves. Loss of FoxC2 leads to defective lymphatic remodelling, lack of valves and increased pericyte coverage [26]. Up-regulation of FoxC2 and subsequently Lyve1 expression mark the maturation of collecting vessels and are followed by the accumulation of basement membrane proteins. Although VEGFR3, Prox1 and FoxC2 remain high in lymphatic valves, their expression recedes in the collecting vessel trunks. Prox1 expression is, however, retained in the entire lymphatic vessel system. Lyve1 expression is almost lost in collecting vessels, but stays high in lymphatic capillaries [27]. Recently, Cx (connexin) 37 and Cx43 were both shown to be required for the formation of lymphatic valves. Both connexins are expressed in embryonic lymphatic endothelium, but later become restricted to LECs upstream and downstream of intraluminal valves. Mice deficient for Cx37 and Cx43 develop lymphoedema, chylothorax and show reflux of lymph [28].

Platelets and myeloid cells in blood–lymphatic vessel separation

A crucial step during the development of the lymphatic vasculature is its separation from the blood circulation, except for the above-mentioned connections to the subclavian veins. Failure of blood and lymph vessels to separate results in lethal haemorrhages and oedema during fetal development. In the adult, formation of illegitimate blood–lymphatic anastomosis causes the development of chylous ascites and fatal peritoneal bleedings [29]. Analysis of mice deficient for the non-receptor Syk (spleen tyrosine kinase), and the adaptors SLP-76 [SH2 (Src homology 2)-domain-containing leucocyte protein of 76 kDa] suggested that blood–lymphatic separation is a lifelong active process [30]. Syk and its downstream signalling mediators SLP-76 and PLCγ (phospholipase Cγ) appear to act in different cell types via distinct mechanisms.

Genetic fate mapping experiments in mouse fetuses identified a Syk-expressing myeloid population that largely...
consists of M2-polarized monocytes. These Syk-expressing cells produce chemokines and growth factors such as VEGF-C and VEGF-D and exhibit a pro-lymphangiogenic activity. In Syk-deficient embryos, this myeloid population massively accumulates in the fetal skin and is characterized by a strongly increased lymphangiogenic activity, resulting in lymphatic hyperproliferation and ultimately the formation of blood–lymphatic shunts [31].

A number of recent studies have demonstrated that, in addition, platelets are crucially involved in the process of blood–lymphatic separation. Carramolino et al. [32] demonstrated erythrocyte-filled lymph sacs in embryos deficient for the homeodomain transcription factor Meis1 (myeloid ecotropic viral integration site 1), which lack megakaryocytes and consequently platelets. Furthermore, targeted ablation of the megakaryocyte lineage reproduced the appearance of blood-filled lymph sacs and, at the same time, excluded defects in the haemopoietic stem cell compartment. Anti-thrombocyte antibody staining located platelets, which interacted with the venous vessel wall close to the sites of nascent lymph sacs [32]. Similarly, Uhrin et al. [33] showed that local activation of platelets in the cardinal vein at sites of newly forming lymph sacs is required for proper blood lymphatic separation. This study proposed that platelets clot in response to endothelial Pdpn and thereby prevent blood from entering the lymphatics. The mucin-type transmembrane glycoprotein T1α/Pdpn is expressed in the cardinal vein around E11 and later in Prox1-positive LECs. Mice lacking this protein die at birth due to respiratory failure, exhibit severe lymphoedema and show blood-filled lymphatics [34]. Pdpn interacts with CLEC-2 (C-type lectin-like receptor 2) on platelets and myeloid cells. Interestingly, CLEC-2-dependent activation of platelets requires the Syk/SLP-76 signalling pathway [35,36].

Regulation of lymph vessel growth by myeloid cells

During pathological conditions, such as tumour growth and inflammation, subsets of myeloid cells are known to promote angiogenesis by producing pro-angiogenic factors [37]. In particular, macrophages display VEGF-dependent and VEGF-independent pro-angiogenic activities [38]. Depending on their polarization state (M1 or M2), macrophages can exert tumour-suppressive or tumour-promoting effects. During inflammation-induced lymphangiogenesis, VEGF-A, -C and -D derived from CD11b+ macrophages play a critical role [39,40]. M2-polarized TEMs (Tie-2-expressing monocytes), which can be recruited by tumour-cell-secreted Ang-2, are potent stimulators of tumour angiogenesis [41]. Inhibition of M-CSF (macrophage colony-stimulating factor), suppresses tumour angiogenesis as well as lymphangiogenesis [42].

Besides providing pro-angiogenic factors, macrophages may also act directly on sprouting vessels. In adipose tissue, VEGF/VEGFR-2-dependent accumulation of Lyve1+ macrophages is crucial for the formation of a dense vessel network [43]. Zumsteg et al. [44] showed that, in vitro, macrophages can form and contribute to lymphatic-like structures. During this process, macrophages appear to incorporate preferentially at tip and branching points, indicating a role in endothelial sprouting [44]. An as yet unidentified role for macrophages in angiogenesis was recently described by Fantin et al. [45], who showed that Tie-2- and NRP1-expressing tissue macrophages can interact with endothelial tip cells and thereby provide a bridge to promote vascular anastomosis.

Imaging lymphatic vessel growth

The formation of new lymphatic vessels through sprouting from an existing vessel bed, as well as the subsequent maturation steps, are highly complex processes, which involve intricate interactions of different cell and tissue types. An in-depth understanding of the regulation of lymph vessels indispensably relies on the ability to image vascular behaviour under various physiological settings. Ideally, optical sectioning is applied which allows the visualization of the spatial arrangement of a vessel bed in a minimally disturbed piece of tissue and is therefore far superior to the traditional analysis of histological sections. The process of sectioning and staining is unavoidable associated with the loss of material and tissue distortion, making subsequent digital reconstruction of the vascular network tedious and time-consuming. Over the last decade, a number of monomeric fluorescent proteins that span a large spectral range from yellow–orange to dark red, have been derived by mutagenesis of mRFP (monomeric red fluorescent protein) [46]. Owing to the superior tissue penetration of red light, red-shifted fluorescent proteins are particularly well suited as genetically encoded markers for different molecules, cells or tissue types [47]. In the context of visualizing vascular structures, the use of fluorescent genetically encoded markers bears significant benefits. Because fluorescent proteins render immunohistochemical staining unnecessary, antibody penetration no longer limits the depth of analysis, which is now only restricted by optical limits. Ultimately, genetically encoded markers should also allow the dynamic imaging of live samples. In addition, the specific marking can be used to isolate or enrich cell populations of interest using FACS.

Imaging genetically encoded fluorescent proteins using two-photon microscopy

A meaningful analysis of vascular structures in living tissue must be powerful enough to obtain data from deep tissue layers (>100 μm), which are not accessible to traditional fluorescence or confocal microscopy. 2P-LSM (two-photon laser-scanning microscopy) provides ideal conditions for deep tissue imaging. Owing to the shortage of endogenous chromophores absorbing in the NIR
Figure 1 | Determination of two-photon biophysical properties of mRFP derivatives

(A) Schematic diagram of the 2P-LSM setup. Fluorophores are simultaneously excited by NIR-pulsed two-photon laser lines at wavelengths of 820 nm and 1100 nm respectively. Reflected fluorescence light was filtered using wavelength-specific bandpass filters and detected by non-descanned (ND) GaAsP (gallium arsenide phosphide) detectors.

(B) Two-photon-excitation spectrum of mOrange determined in transfected Cos-1 cells expressing different levels of protein.

(C) Two-photon-excitation spectra of various mRFP derivatives determined in transfected Cos1 cells. Maximal fluorescence intensities of the different proteins were normalized to 1.

(D) Two-photon-bleaching curves for several fluorescent proteins, as measured by fluorescence decay after illumination with 1100 nm NIR-pulsed two-photon laser light in transfected Cos-1 cells and plotted as normalized intensities against total exposure time. Half-time was determined as 50% of initial intensity. mOrange2 exhibits a 4-fold greater photostability than mOrange. rel. intensity, relative intensity; a.u., arbitrary units.

(near-IR), the NIR two-photon excitation light provided by femtosecond pulsed TiSa (titanium–sapphire) lasers is less scattered, penetrates deeper and causes less photobleaching and toxicity in tissue compared with the visible light used in classical far-field fluorescence microscopy [48].

For intravital imaging, we use a custom 2P-LSM designed by LaVision BioTec, which is equipped with two TiSa lasers, one of which is directly routed into the scan optics. The second TiSa laser is coupled to an OPO (optical parametric oscillator) resulting in a wavelength extension that provides femtosecond pulsed-IR light in the range 1070–1250 nm [49] (Figure 1A). To determine the two-photon imaging parameters of various mRFP derivatives, we measured their two-photon excitation spectra. Surprisingly, we found the optimal excitation wavelength for the mRFP derivatives mStrawberry, mOrange, mCherry and mPlum, but also for TurboFP635, to be approximately 1100 nm, while excitation below 1050 nm was negligible (Figures 1B and 1C). Our findings have two important implications. First, because NIR light with wavelength greater than 1050 nm is not efficiently generated by TiSa lasers and hence excitation efficiency for mRFP derivatives is suboptimal, they emphasize the vital importance of OPO-based 2P-LSM. Secondly, because of the unexpected property that all mRFP derivatives are optimally excited at 1100 nm, multicolour imaging is immediately possible, as long as the emission signals can be separated satisfactorily. In addition, an analysis of the photostability of the mRFP derivatives demonstrated significantly decreased bleaching of mOrange2, resulting in a more than doubled fluorescence half-life compared with its predecessor mOrange (Figure 1D). Similar results have recently been reported using OPO-based 2P-LSM for the...
Figure 2 | Confirmation of lymphatic-specific expression of the fluorescent reporter protein mOrange2 in adult and fetal prox1-mOrange2-pA-BAC mice

(A) A cDNA encoding mOrange2–SV40pA (simian virus 40 polyadenylation site) and an ampicillin-resistance cassette for positive selection were introduced at the Prox1 start codon into the BAC clone RP23-190F21 replacing part of exon 2 using Red/ET recombination. (B) Endogenous expression of prox1-mOrange2 in lymph vessels of the skin imaged by fluorescence stereomicroscopy and in the ear imaged by confocal laser-scanning microscopy. (C) Skin whole mount of a prox1-mOrange2-pA-BAC embryo (E14.5) counterstained with positively identifying markers. The vascular marker PECAM1 is expressed in blood vessels and more weakly in lymphatic vessels, whereas Lyve1 is expressed exclusively in lymphatic vessels. Fl., fluorescence.

Generating a reporter mouse for the visualization of lymphatic vessels

In combination with GFP (green fluorescent protein), regulatory elements of the Tie-2 and VE-cadherin promoter regions have been successfully used to visualize blood vessels in the mouse [52,53]. We wanted to generate a mouse model for the visualization of lymphatic vessels that can be combined with existing genetically labelled mice already bearing either green- or red-labelled cells. We decided to use the Prox1 promoter to drive expression of the mOrange2 protein in lymphatic endothelium. Prox1-driven expression is particularly suitable for monitoring lymph vessels both in embryos and adults, because Prox1 serves as a master regulator of lymphatic differentiation during embryonic lymphangiogenesis and its expression is maintained also in adult LECs [27]. The mOrange2 protein is spectrally sufficiently distinct from green and red fluorescent proteins to be optically separated by narrow-bandpass filters and its increased photostability makes it a good choice for long-term observations in vivo.

We used recombineering in bacteria to generate a BAC (bacterial artificial chromosome) construct comprising approximately 100 kb upstream and downstream of the Prox1-initiating ATG, to which the mOrange2 cDNA was fused in-frame (Figure 2A). Transgenic mice harbouring this construct, prox1-mOrange2-pA-BAC mice, showed faithful labelling of lymphatic capillaries in various organs, including mesentery, skin and lymph nodes (Figures 2B
Figure 3 | Intravital two-photon imaging of lymphatic vessels

(A) Superficial lymphatic vasculature of the fetal skin (E16.5) imaged using 2P-LSM. Superficial lymphatics (marked by mOrange2 expression) show different developmental and remodelling stages of fetal lymphangiogenesis. (B) 2P-LSM intravital microscopy of lymphatic valve action. A series of images is presented showing the opening of a lymphatic valve upon lymph flow in an adult mouse. Lymphatic vessels were marked by mOrange2 expression and the surrounding tissue is visualized through second harmonic signals from the extracellular matrix. (C) Lymphatic valve architecture and lymph flow after FITC injection. Two-photon images visualize a lymphatic collecting vessel including a saddle-shaped lymphatic valve (left-hand panel, red) and FITC uptake into lymphatic vessels (central panel, green content within the vessel) embedded in abundant connective tissue (right-hand panel, merged image showing FITC-filled lymphatic vessel and surrounding tissue).

and 2C). During the course of our analysis, a Prox1-promoter driven GFP reporter model was published that showed a very similar, if not identical, expression pattern [54].

Intravital 2P-LSM of proxl-mOrange2-pA-BAC mice finally allows us to visualize the superficial lymphatic vessel network in developing mouse embryos (Figure 3A), as well as lymphatic vessels and valve movement in the skin of adult mice (Figures 3B and 3C). This approach offers, for the first time, the unique opportunity to visualize the growth and formation of lymphatic vessels in a living organism and promises for the near future exciting, so far inaccessible, insights into the biology of the lymphatic vessel system.
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