Modeling lymphangiogenesis in a 3D-culture system

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ABSTRACT

Unraveling the molecular mechanisms of lymphangiogenesis is hampered by the lack of appropriate *in vitro* models of three-dimensional (3D) lymph vessel growth which can be used to exploit the potential of available transgenic mice. We developed a potent reproducible and quantifiable 3D-culture system of lymphatic endothelial cells, the lymphatic ring assay, bridging the gap between 2D-*in vitro* and *in vivo* models of lymphangiogenesis. Mice thoracic duct fragments are embedded in a collagen gel leading to the formation of lymphatic capillaries containing a lumen as assessed by electron microscopy and immunostaining. This assay phenocopies the different steps of lymphangiogenesis, including the spreading from a preexisting vessel, cell proliferation, migration and differentiation into capillaries. Our study provides evidence for the implication of an individual matrix metalloproteinase, MMP-2, during lymphangiogenesis. The lymphatic ring assay is a robust, quantifiable and reproducible system which offers new opportunities for rapid identification of unknown regulators of lymphangiogenesis.

Blood and lymphatic vessels form a contiguous system for the circulation of fluids and the delivery of molecules and cells within the body. Despite its implication in numerous pathologies such as lymphedema and cancer[1], the lymphatic system has until recently been overshadowed by the greater emphasis placed on the blood vascular system. Understanding the molecular and cellular basis of the functional lymphatic abnormalities associated with different diseases is essential for the development of novel therapeutic strategies[2]. Studies on lymphangiogenesis is hampered by difficulties in culturing lymphatic capillaries as three-dimensional structures *in vitro* that mimic the *in vivo* features of lymphatic vessels and lymphangiogenesis.

The *in vivo* models of lymphangiogenesis include (1) the induction of tumor-associated lymphangiogenesis by VEGF-C overexpresion in tumor cells[3] or in transgenic mice[4], (2) lymphatic cell hyperplasia induced by intraperitoneal injection of incomplete Freund's adjuvant[5,6], (3) lymphedema induced by the excision of a circumferential band of skin in mouse tail[7] or microsurgical ablation of tail lymph vessels[8] and (4) the corneal assay in which growth factor-containing pellets are implanted into corneal micropockets[9]. In addition to these murine models, the *Xenopus* tadpole is a recently described genetic model with which to investigate lymphangiogenesis[10]. One of the main limitations of using many of these models to identify key regulators of lymphangiogenesis is the involvement of inflammatory reactions, thus making discrimination between a direct effect on lymphatic endothelial cells and an indirect effect caused by the modulation of inflammatory mediators difficult.

Several lymphatic 2D-culture systems now available use human dermal lymphatic cells isolated either by immuno-purification with Fluorescence-Activated Cell Sorting (FACS) or by magnetic beads[11], and on occasion employ transformation with human telomerase reverse transcriptase (hTERT-HDLEC)[12]. Thoracic duct endothelial cells from

different species are isolated by enzymatic digestion[13,14]. Lymphatic endothelial cell cultures are also derived from lesions induced by injecting Freund's Adjuvant into mice[5,6]. In addition, lymphatic endothelial cell differentiation are induced in embryoid bodies[15]. These systems suffer from several common drawbacks that include (1) the limited number of cells that can be obtained by isolating non-transformed cells, (2) the non-physiological features of immortalized cells, and (3) the putative dedifferentiation of cells in 2D-cultures. Moreover, none of these culture systems adequately represents 3D-growth of lymphatic microvessels with a lumen. A model that phenocopies the different steps of lymphangiogenesis, including the spreading from a preexisting vessel, cell proliferation, migration and differentiation into capillaries is therefore urgently needed to overcome these obstacles in order to gain new insights into the molecular mechanisms of lymphangiogenesis[2].

The aortic ring assay is frequently used to investigate the molecular basis of angiogenesis[16-18], to exploit the numerous genetically-modified mice that are generated[17], and to evaluate the pharmacological efficacy of pro- and anti-angiogenic agents[18]. In this study, we have successfully adapted the aortic ring model to the murine thoracic duct and developed a computerized method of quantification. These reproducible 3D-lymphatic cultures have been validated as a new model that provides new insights into the mechanisms that underlie lymphangiogenesis, and allows the identification of candidate genes and molecules involved in this disease-relevant process. The lymphatic ring assay bridges the gap between *in vitro* and *in vivo* studies and allows genetic analysis by using thoracic ducts from genetically-modified mice. In the present study, we also addressed the possibility that matrix metalloproteinases (MMPs) which are recognized as important actors of angiogenesis[19] could also modulate lymphangiogenesis. We provide evidence that MMP-2 is a regulator of lymphangiogenesis.

RESULTS

Estabblishment of the lymphatic ring assay

To develop 3D-lymphatic ring cultures, we excised thoracic ducts from mice and dissected it transversely to generate lymphatic duct rings, which were then embedded into type I collagen gels (**Fig. 1)**. Five days later, these lymphatic ring cultures exhibited outgrowth of cells that organized into capillary-like structures. Lymphatic vessel outgrowth from thoracic duct rings was stimulated in a dose-dependent manner by serum, and was never detected in its absence (**Fig. 2a**). Incubation of lymphatic rings in a more physiologically relevant atmosphere (5% O_2) significantly accelerated capillary formation ($P<0.05$) and improved the percentage of explants exhibiting outgrowth of capillary-like structures, which after 11 days of incubation was 55% ± 6% under ambient oxygen culture conditions and reached 87% ± 4.5% when 5% O_2 was employed (**Fig. 2c**).

To measure lymphatic capillary outgrowth from thoracic duct rings, we developed a method to quantify objectively the lymphatic sprouts and their interconnected network by computer-assisted image analysis. After image binarization, lymphatic vessel distribution was determined by measuring the number of intersections of microvessels with a grid obtained by drawing concentric rings around the ring boundaries (represented in **Fig. 2a**). This computerized analysis confirmed the progressive outgrowth of capillary structures from day 7 to day 14, as well as the enhancement of lymphatic vessel formation when 5% O_2 was employed (**Fig. 2b, c**). While no difference between vessel outgrowth under 5% O_2 and 21% O_2 was observed during the first week of culture, improvement of lymphatic vessel outgrowth under 5% O_2 conditions started on day 9 and continued until the end of the assay ($P<0.05$). From day 7 to day 9, these vessels became progressively more interconnected as

demonstrated by the progressive displacement from the explant of the maximal number of endothelial cells.

For all subsequent assays, we cultured lymphatic rings in defined optimal conditions (10% FCS and 5% O_2) leading to outgrowth of 87% ± 4.5% of the rings and a quantifiable vessel network.

Presence of typical features of lymphatic vessels

Immunochemical characterization of cells growing out from thoracic duct rings revealed positive staining for LYVE-1 (**Fig. 3a**) and VEGFR-3 (**Fig. 3b**), two markers of lymphatic vessels[2]. Cells were positive for Von Willebrand Factor (**Supplementary Fig. 1** online), supporting their endothelial origin. Cells were negative for markers of other cell types such as blood endothelial cells (CD34), fibroblasts (Thy1.1) and inflammatory cells (CD45) (**Supplementary Fig. 1** on line). Less than 15% of spreading cells, probably smooth muscle cells, were positive for alpha Smooth Muscle Actin (aSMA) (**Supplementary Fig. 1** on line). However, none of these cells covered the lymphatic capillaries. They were localized around the rings and did not participate in the structure of the vascular network. Therefore, despite the fact that the starting material is composed of different cell types, the cells forming the vascular network are exclusively lymphatic endothelial cells.

Analysis by transmission electron microscopy revealed microvessels composed of a single layer of cells delimiting a lumen (**Fig. 3 c,d**). These endothelial cells contained Weibel Palade Bodies (**Fig. 3g**) and had overlapping boundaries without well-established adhesive junctions, but with occasional tight junctions (**Fig. 3f**). Intercellular junctions were formed by simple abutment of apposing endothelial cells or by interdigitation of endothelial cell margins (**Fig. 3e**). The lymphatic endothelial cells also displayed a polarized organization characterized by a regular abluminal surface apposed to the surrounding matrix and cell

processes extending into the lumen on the luminal side (**Fig. 3c**). All these features are typical of lymphatic vessels[20].

Sensitivity to lymphangiogenic factors

We validated the lymphatic ring assay as a model for lymphangiogenesis by evaluating the impact of a well-recognized lymphangiogenic factor (VEGF-C)[2] on lymphatic endothelial cell outgrowth. As VEGFs are present in serum, we supplemented the culture medium with a chemically-defined serum (Ultroser) at a concentration of 4%, instead of FCS. The basal level of lymphatic cell sprouting observed under these conditions was improved by adding VEGF-C to the medium (2.7-fold improvement at the optimal concentration of 10 ng/ml) ($P<0.05$) (**Fig. 4a**) (**Supplementary Fig. 2** online). Furthermore, a soluble form of VEGFR-3 inhibited the induction of microvessel outgrowth by VEGF-C ($P<0.05$) (**Fig. 4b**). Moreover, MAZ51, an inhibitor of ligand-induced VEGFR-3 autophosphorylation[21], also reduced serum-dependent lymphatic outgrowth (52% reduction at the optimal concentration) ($P<0.05$) (**Fig. 4c**).

Suitability to screen for lymphangiogenic factors

To identify other lymphangiogenic factors, we tested recombinant growth factors in the lymphatic ring assay under serum-free conditions in the presence of 4% Ultroser. We first tested Placental Growth Factor-1 (PlGF-1), a member of the VEGF family, and Fibroblast Growth Factor-2 (FGF-2) which have been implicated in pathological angiogenesis (**Fig. 4d and e** and **Supplementary Fig. 3** on line). Although both factors used at 5 ng/ml slightly increased lymphatic cell outgrowth, this increase was not statistically significant ($P>0.05$) and higher concentrations did not affect lymphatic endothelial cell outgrowth (**Fig. 4d and e**). In agreement with these results, suramin, a polysulfonated naphthylurea that binds to and

inhibits FGF[22], was unable to inhibit lymphatic outgrowth in 10% FCS lymphatic ring cultures (**Fig. 4f**). In sharp contrast, Platelet Derived Growth Factor-BB (PDGF-BB) at 5 ng/ml induced a three-fold increase in lymphatic vessel formation ($P<0.05$) (**Fig. 4g** and **Supplementary Fig. 3** online). This data supports previous studies reporting a lymphangiogenic effect of PDGF-BB. PDGF-BB has been shown to induce intratumoral lymphangiogenesis and lymphatic metastasis *in vivo*, and to stimulate lymphangiogenesis in corneal assays[9,23]. An inhibitor of the PDGF receptor abrogated lymphatic cell outgrowth stimulated by 5 ng/ml PDGF ($P<0.05$) (**Fig. 4h**) and reduced serum-induced lymphangiogenesis in a dose-dependent manner (59% reduction at 3.5 µg/ml) ($P<0.05$) (**Fig. 4i**). Cell viability assays at the end of the experiment showed that this inhibitor did not exert a toxic effect. Together these data show that PDGF-BB but not PlGF-1 and FGF-2, is a lymphangiogenic factor acting directly on lymphatic endothelial cells.

Analysis of proteases implicated in lymphangiogenesis

A major potential application of the lymphatic ring model is the use of thoracic ducts derived from genetically modified mice to assess the role of candidate genes in lymphangiogenesis. Since we have previously demonstrated that PAI-1 deficiency impairs blood endothelial cell migration from aortic rings[24], we investigated whether PAI-1 plays a similar role in lymphangiogenesis. Lymphatic cell outgrowths were equivalent from thoracic duct fragments derived from PAI-$1^{-/-}$ mice or wild type littermates (**Fig. 5a and Supplementary Fig. 4** on line). Moreover, aprotinin, a serine protease inhibitor, did not impair lymphangiogenesis in these assays (**Fig. 5b**). These results indicate that PAI-1 is dispensable for migration and outgrowth of lymphatic endothelial cells, in contrast to its important role in angiogenesis.

To address the putative role of metalloproteinases in lymphangiogenesis, we first examined whether MMPs are present in the conditioned medium from lymphatic ring assays. Gelatinolytic zymographic analysis revealed the presence of two pro-Mmp-2 species[25] without any active form (**Fig. 5f**). Mmp-2 activation likely occurs at the cell surface in the collagen matrix and is thus not detectable in the supernatant. TIMP-2, a physiological inhibitor of MMPs, induced a dose-dependent decrease in lymphatic network formation (55% reduction at 1 µg/ml) ($P<0.05$) (**Fig. 5c**). Furthermore, RO-28-2653, a synthetic inhibitor targeting mainly MMP-2, MMP-9 and MMP-14[18], inhibited lymphatic vessel outgrowth ($P<0.05$) (**Fig. 5d**). Most importantly, thoracic duct rings derived from Mmp-$2^{-/-}$ mice exhibited impaired lymphangiogenesis in comparison to those from wild type mice (**Fig. 5e**). Together, these data establish MMP-2 as an important effector of lymphangiogenesis.

DISCUSSION

We describe a reproducible and quantifiable system for cultivating lymphatic endothelial cell microvessels in 3D-lymphangiogenesis assays. Lymphatic capillaries obtained from rings of murine thoracic lymphatic ducts embedded in a collagen gel were formed by a single layer of endothelial cells with overlapping boundaries and expressed specific markers of lymphatic vessels. The absence of contaminating cells in the lymphatic network such as blood endothelial cells, fibroblasts, smooth muscle cells and inflammatory cells was verified by whole mount immunostaining.

In vitro 2D-cultures using isolated lymphatic endothelial cells are useful assays for studying cell proliferation[11]. In addition, tube forming assays on a 3D-matrix[5] or in embryoid bodies[15] are very potent models to investigate cell migration and tube-like structure formation. However, tube formation in these assay reflects the capacity to generate a complex plexus similar to that observed during embryogenesis[26] and therefore mimics vasculogenic processes rather than lymphangiogenesis defined as the formation of new lymphatic vessels from pre-existing ones. Although no data is yet available demonstrating that *in vivo* lymphatic endothelial cells sprout from the adult thoracic duct, the present lymphatic ring assay has the advantage of recapitulating different steps of lymphangiogenesis, including endothelial cell sprouting from an existing vessel, migration and differentiation into capillaries.

Studies in 3D-cultures are often hampered by difficulties in achieving objective and reproducible quantification. Many quantification strategies are based on the manual processing of images which is time-consuming and subjective[27]. To overcome this limitation, a novel computer-assisted method of image analysis has been created to quantify objectively the lymphatic vascular network. The culture model and its computerized quantification were validated by testing well-known regulators of lymphangiogenesis and angiogenesis. The involvement of the VEGF-C/VEGFR-3 pathway[2] was supported by stimulation of lymphatic

endothelial cell outgrowth by VEGF-C, and its inhibition by a soluble form of VEGFR-3 or MAZ51 which blocks ligand-induced VEGFR-3 autophosphorylation[21]. In our system, PlGF or FGF-2 did not affect lymphatic capillary formation. Accordingly, suramin, an FGF trapping molecule[22], did not inhibit serum-stimulated lymphangiogenesis. Therefore, PlGF and FGF-2 do not directly control lymphatic endothelial cell outgrowth and are not sufficient *per se* to promote sprouting from pre-existing vessels. PlGF[28] and FGF-2 probably exert lymphangiogenic effects *in vivo* indirectly by promoting the release of pro-lymphangiogenic factors by accessory cells. For instance, FGF-2 influences lymphatic cell growth in mouse cornea assays by promoting VEGF-C secretion from blood vascular endothelial cells[29].

Our data confirmed the lymphangiogenic activity of PDGF-BB[9,23], which could be abrogated by an inhibitor of PDGF receptor tyrosine kinase. Interestingly, blockade of the PDGF-receptor was sufficient to inhibit serum-stimulated lymphatic outgrowth. This identifies PDGF as a potent serum-derived lymphangiogenic factor.

A major potential application of the lymphatic ring assay is the investigation of the impact of gene deficiency on lymphangiogenesis through the exploitation of genetically modified mice. As proof of principle we examined the role of proteases and their inhibitors in lymphangiogenesis. Although PAI-1 is essential for blood endothelial cell migration in the aortic ring assay[24], we provided evidence here that PAI-1 is dispensable for lymphatic endothelial cell outgrowth. In contrast, lymphangiogenesis was reduced by physiological MMP inhibitor and a broad spectrum synthetic MMP inhibitor. Lymphangiogenesis was also impaired by the specific targeted deletion of *Mmp-2*, while blood endothelial cell outgrowth in the aortic ring assay was not affected in Mmp-2$^{-/-}$ [19]. Although the contribution of MMPs to lymphangiogenesis has been suggested through the use of broad spectrum inhibitors[6], our study suggests that MMP-2 is a regulator of this process. These data are consistent with the kinetics of Mmp-2 production observed in a lymphangiogenic model of adult skin

regeneration where Mmp-2 expression closely correlates with lymphatic endothelial cell infiltration[7]. By identifying PAI-1 as dispensable and MMP-2 as indispensable factors for lymphangiogenesis, we have demonstrated that the lymphatic ring assay is a powerful tool for delineating the difference between molecular mechanisms underlying angiogenesis and lymphangiogenesis.

In conclusion, the lymphatic ring assay is an innovative system for the 3D-cultivation of murine lymphatic microvessels. This system allows (1) identification of novel candidates that regulate post-natal growth and maintenance of lymphatic vasculature, (2) screening for pro- and anti-lymphangiogenic agents, (3) exploitation of genetically modified mice, and (4) identification of specific mechanisms that underlie lymphangiogenesis or angiogenesis. The combination of this model with computerized quantification offers an unprecedented potential for gaining new insights into lymphangiogenesis, with important prospects for the design of strategies for the manipulation of the lymphatic system to counteract diseases such as metastatic cancer and lymphedema.

METHODS

Thoracic duct collection and three-dimensional lymphatic ring cultures

For the setting up of the assay, we identified thoracic ducts by intradermal injection of Evans blue (1.5% PBS) into the paws and ears of donor mice before sacrifice (**Fig. 1b**). For routine assays, Evans blue staining was not required and dissection was performed without staining as illustrated in **Supplementary Fig. 5** online. We cultured thoracic ducts from male and female C57Bl/6J mice 2 to 4 months of age. We used the previously described PAI-1-deficient mice (PAI-$1^{-/-}$)[24], Mmp-2-deficient mice (Mmp-$2^{-/-}$)[19], and the corresponding wild-type mice (PAI-1 WT and Mmp-2 WT). All experiments using live mice were approved by the local ethical committee of the University of Liège.

We prepared cylindrical agarose wells prior to animal sacrifice and lymphatic duct excision[16,17,30]. A sterile 1.5% type VII agarose solution (Sigma-Aldrich, St Louis, MO) was heated, poured in a 100mm culture dish (25ml/dish) (BD Falcon, New Jersey, USA) and allowed to gel at 4°C. We prepared agarose cylinders by punching two concentric circles in the agarose with punchers of 10 and 17mm diameter and transferred to a 86x12 Bacterial Petri Dish (Nunc, Roskilde, Denmark). We obtained thoracic ducts by microsurgery using very thin microdissecting forceps and Vannas scissors (**Supplementary Fig. 5** online). The periaortic fibroadipose tissue was first carefully dissected (step 1) (**Supplementary Video 1** on line). We removed fat tissue surrounding the lymphatic ring and took care not to damage the lymphatic wall (step 2) (**Supplementary Videos 2 and 3** on line). During this dissection, we continuously filled with sterile PBS the thoracic cavity bounded by the rib cage closed and the intact diaphragm. After cutting its edges (step 3) (**Supplementary Video 4** on line), the duct was immediately transferred to a culture dish containing ice-cold serum-free MEM media (Invitrogen, Carlsbad, CA) and cut into 1 mm-long rings (about 10 pieces per duct) (**Supplementary Fig. 5** on line). We then embedded ring-shaped explants between two layers

of rat tail interstitial collagen gel polymerized in cylindrical agarose wells prepared as described above. We obtained collagen gels by mixing 7.5 volumes of Collagen R (2 mg/ml) (Serva Electrophoresis, Heidelberg, Germany) with 1 volume of 10x MEM (Gibco, Grand Island, NY), 1.5 volume of $NaHCO_3$ (15.6 mg/ml) and approximately 0.1 volume of NaOH (1 M) to adjust the pH to 7.4. Four gels, each one containing a lymphatic duct ring, were maintained in a culture dish with 6 ml of MCDB131 (Invitrogen, Carlsbad, CA) supplemented with 25 mM $NaHCO_3$, 10% fetal calf serum (FCS) (Gibco, Grand Island, NY) or 4% Ultroser G (BioSepra, Cergy Saint Cristophe, France), 1% glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. We kept cultures at 37°C in a humidified incubator (HERAcell 150, Heraeus, Hanau, Denmark) under ambient (21% O_2, 5% CO_2, 74% N_2) or reduced oxygen conditions (5% O_2, 5% CO_2 and 90% N_2). We used autologuous mouse serum (3%) for the maintenance of lymphatic ring cultures derived from PAI-$1^{-/-}$, Mmp-$2^{-/-}$ mice or their corresponding wild type counterparts.

Treatment with growth factors and inhibitors

We prepared lymphatic rings as indicated above and maintained it in MCDB131 medium supplemented with either 4% Ultroser (BioSepra, Cergy Saint Cristophe, France) or 10% FCS (Gibco, Grand Island, NY). We added the following factors to the culture medium at the beginning of the experiment as appropriate: recombinant mouse VEGFR-3/Fc chimera (sVEGFR-3) (R&D Systems, Minneapolis, MN), human Placental Growth Factor-1 (hPlGF-1, R&D Systems, Minneapolis, MN), human Fibroblast Growth Factor-2 (hFGF-2, R&D Systems, Minneapolis, MN), Suramin (Sigma-Aldrich, St Louis, MO), human Platelet-Derived Growth Factor BB (hPDGF-BB, R&D Systems, Minneapolis, MN), PDGF Receptor Tyrosine Inhibitor II (Calbiochem, San Diego, CA), and Aprotinin (Roche, Basel, Switzerland). VEGF-C, MAZ51, Ro-28-2653 and rTIMP-2 have been described[18]. We used a

Viability/Cytotoxicity kit (Molecular Probes, Engene, OR) according to manufacturer's instructions to assess the potential toxic effects of these compounds.

Quantification of lymphangiogenesis

Images for analysis were taken under identical conditions of light, contrast and magnification. We kept processing parameters constant to allow automatic image processing. We obtained binary images in a two-step procedure as described[27] and illustrated in **Supplementary Fig 6** online. We determined the number of intersections of microvessels with a grid of concentric rings drawn by making successive dilatations at similar intervals of the thoracic duct boundary. The number of intersections between microvessels and this grid was determined for each image. Microvessel distribution is defined as the number of intersections plotted as a function of the distance from the ring. We considered at least five images per condition, yielding five microvessel distribution curves per condition. We then obtained a mean microvessel distribution curve by averaging individual distributions point by point, and smoothed using cubic spline interpolation. For comparison, curves were normalized, taking the maximum of the control curve as 1. From the distribution curves obtained from individual images, we determined the following parameters: (a) the mean longest length reached for microvessels, which corresponds to the maximal distance of vessel migration, and (b) the mean largest number of microvessel intersections. To determine the statistical significance of differences observed between control distribution curves and those of experimental conditions, we used a Student's *t*-test to calculate P-values for experimental values obtained 0.25 mm, 0.50 mm, 1.0 mm and 1.5 mm from the thoracic duct rings. Image analysis algorithms used in this work were applied with the software Aphelion (3.2) from Adcis (France). We performed statistical analysis using the statistics toolbox of Matlab 7.0.

Additional Methods.

Descriptions of gel whole-mount immunostaining, transmission electron microscopy and gelatinolytic zymography are available in **Supplementary Methods** online.

LEGENDS OF FIGURES

Figure 1: Lymphatic ring assay. (**a**) Schematic representation of the assay. Lymphatic thoracic ducts resected from mice were cut into small pieces, which were then embedded in type I collagen gel. Quantification was performed using computerized image analysis by determining the number of intersections of vessels with a grid obtained by drawing concentric rings around the ring boundaries. Two representative curves are shown to illustrate the method. (**b**) Identification of lymphatic thoracic duct *in vivo* by Evans blue staining. Evans blue (5% in PBS) was injected in the ears and paws of donor mice. After 15 min, the blue-stained lymphatic thoracic duct could be distinguished from the unstained blood vessels. (**c-d**) Immunostaining of lymphatic and aortic rings with anti-LYVE-1 antibody revealed positive staining for the lymphatic duct (black arrow heads), but not for the aorta, which served as a negative control. Scales Bars (**a-b**): 1mm, (**c**): 10µm and (**d**): 200µM.

Figure 2: Lymphatic endothelial cell outgrowth from mouse thoracic duct. (**a**) Lymphatic duct explants embedded in type I collagen gel were cultured for 9 days in medium supplemented with increasing concentrations of fetal calf serum (FCS, 0 to 20%), either under ambient or reduced oxygen conditions (21% O_2 and 5% O_2, respectively). An example of binary image processing of lymphatic rings is shown in the black boxes (panel **a**, lower right) for rings cultured with 10% FCS under 21% O_2 (left) or 5% O_2 (right). A grid obtained by dilatations of ring boundaries was used to quantify intersections of the grid with vessels (number of intersections between microvessels and quantification grid) using computer-assisted image analysis. (**b**) Kinetic study of lymphatic microvessel outgrowth from day 7 to day 14. The number of intersections is plotted as a function of distance to the ring. (**c**) Percentage of rings exhibiting outgrowth of capillary-like structures cultured under 21% O_2 or

5% O_2 at different time points. Data are mean ± s.e.m. of 4 separate experiments, each running at least 5 rings. *: $P<0.05$

Figure 3: Characterization of three-dimensional lymphatic ring cultures. Outgrowing lymphatic endothelial cells were immunopositive (in green) for (**a**) LYVE-1 and (**b**) VEGFR-3. Red staining corresponds to nuclear staining with propidium iodide. Scale bar in **a-b**: 40 µm. (**c-g**) Representative electron micrographs showing: (**c, d)** endothelial cells delimiting a lumen and surrounded by collagen matrix, (**e**) overlapping and interdigitating terminal margins of adjacent endothelial cells, (**g**) Weibel Palade Bodies, and (**f**) tight junction (corresponding to higher magnification of the insert in **e**). Panels **e** and **g** correspond to higher magnifications of areas delimited by insets in **d**. Scale bars in **d-g**: 1 µm.

Figure 4: Modulation of lymphatic vessel outgrowth by growth factors and inhibitors. To evaluate the pro-angiogenic effect of growth factors, lymphatic rings were cultured in Ultroser-supplemented medium (**a, d, e, g**). For inhibition assays, control medium consisted of standard medium containing 10% FCS (**c, f, i**) or Ultroser-supplemented medium containing the targeted growth factor (**b, h**). Cultures were treated with increasing concentrations of (**a**) VEGF-C, (**b**) sVEGFR-3 in the presence of 10 ng/ml VEGF-C, (**c**) MAZ51 to rings cultured with 10% FCS (**d**) PlGF, (**e**) FGF-2, (**f**) suramin (FGF trapper), (**g**) PDGF, (**h**) PDGF receptor (PDGF-R) inhibitor in the presence of 5ng/ml PDGF or (**i**) with 10% FCS. Quantification was performed by computerized analysis and curves were normalized, taking the maximum of the control curve as 1, as described in methods. Each curve presented is a mean microvessel distribution curve obtained by averaging the five individual distributions generated for each experimental condition (n =5). A Student's *t*-test is

used to determine the statistical differences between control distribution curve and curves of experimental conditions. *: P<0.05; NS= not significant.

Figure 5: Modulation of lymphatic vessel outgrowth by proteases and their inhibitors. Lymphatic rings cultured in the presence of serum (FCS) (**a, c, d**) were treated with increasing concentrations of (**a**) aprotinin, (**c**) TIMP-2, or (**d**) synthetic MMP inhibitor (RO-28-263). Lymphatic rings cultured in the presence of autologous mouse serum derived from (**b**) $PAI^{-/-}$ mice, (**e**) $Mmp\text{-}2^{-/-}$mice and (**b, e**) the corresponding wild type mice (WT). Computerized quantification was performed and curves were normalized as described in materials and methods. Each curve presented is a mean microvessel distribution curve obtained by averaging the five individual distributions generated for each experimental condition (n =5). A Student's *t*-test was used to determine the statistical differences between control distribution curve and curves of experimental conditions. *: P<0.05; NS = not significant. Gelatin zymography was performed on medium conditioned by mouse lymphatic rings (LR) issued from wild type mice (WT) or MMP2-deficient mice ($MMP2^{-/-}$) during 11 days of culture (**f**). Medium conditioned by HT1080 cells (HT) was used as a control to identify Mmp-2 and Mmp-9.

LIST OF SUPPLEMENTARY ITEMS

Supplementary Figure 1: Characterization of three-dimensional lymphatic ring cultures.

Supplementary Figure 2: Effect of VEGF-C pathway on lymphatic endothelial cell outgrowth.

Supplementary Figure 3: Effect of growth factors on lymphatic endothelial cell outgrowth.

Supplementary Figure 4: Effect of proteolytic pathway on lymphatic endothelial cell outgrowth.

Supplementary Figure 5: Visualization of lymphatic thoracic duct excision.

Supplementary Figure 6: Illustration of the method of quantification.

Supplementary Methods

Supplementary Video 1-4 : Lymphatic thoracic duct dissection.

Thoracic ducts were obtained by microsurgery using very thin microdissecting forceps and Vannas scissors. The periaortic fibroadipose tissue was first carefully dissected. Fat tissue surrounding the lymphatic ring was removed and care was taken not to damage the lymphatic wall. Both edges were then cut and the duct was immediately transferred to a culture dish.

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