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VEGF-C Induces Lymphangiogenesis and Angiogenesis in the Rat Mesentery Culture Model

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Abstract

Objective—Lymphatic and blood microvascular systems are critical for tissue function. Insights into the coordination of both systems can be gained by investigating the relationships between lymphangiogenesis and angiogenesis. Recently, our laboratory established the rat mesentery culture model as a novel tool to investigate multicellular interactions during angiogenesis in an intact microvascular network scenario. The objective of this study was to determine whether the rat mesentery culture model can be used to study lymphangiogenesis.

Methods—Mesenteric tissue windows were harvested from adult male Wistar rats and cultured for 3 or 5 days in either serum-free minimum essential media (MEM) or MEM supplemented with VEGF-C. Tissues were immunolabeled for PECAM and LYVE-1 to identify blood and lymphatic endothelial cells, respectively. Tissues selected randomly from those containing vascular networks were quantified for angiogenesis and lymphangiogenesis.

Results—VEGF-C treatment resulted in an increase in the density of blood vessel sprouting compared to controls by day 3. By day 5, lymphatic sprouting was increased compared to controls.

Conclusions—These results are consistent with *in vivo* findings that lymphangiogenesis lags angiogenesis after chronic stimulation and establish a tool for investigating the interrelationships between lymphangiogenesis and angiogenesis in a multi-system microvascular environment.

Keywords

Lymphangiogenesis; angiogenesis; microcirculation; microvascular network

Introduction

Lymphatic and blood microvascular networks play critical roles in the trafficking of leukocytes and the clearance of excess fluid from local tissue spaces [23,38]. Given the importance of these dynamics in inflammation, wound repair, tumor metastasis, lymphedema, and other pathological conditions, understanding lymphatic/blood vascular patterning in adult tissues is necessary [17,28,41,46]. Mis-patterning between growing lymphatic and blood vessels is in general thought to lead to tissue dysfunction. However, most studies focus on either angiogenesis, defined as the growth of new blood vessels from

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existing ones, or lymphangiogenesis, defined as the analogous growth of new lymphatic vessels. The lack of a model that allows mechanistic investigation of both vessel types at the same time has caused lymphatic/blood vascular patterning to remain poorly understood.

Common *in vitro* models for angiogenesis include 2-dimensional culture systems, 3-dimensional culture systems, and blood vessel explant assays (i.e. aortic ring assay) [21]. A common critique of these models is that they do not allow for investigation of mechanistic interactions between multiple cell types in a real microvascular network scenario. Capillary sprouting in the aortic ring assay originates radially from an aorta slice and not microvessels where angiogenesis normally occurs. Even fewer models exist for investigating lymphangiogenesis. The lymphatic ring assay (analogous to the aortic ting assay) was introduced in 2008 [7] and enables the quantification of lymphatic sprouting through a three dimensional scaffold from a slice of a thoracic duct, but, as is the case for the aortic ring model, sprouting does not occur off microvessels. To our knowledge, a model does not exist for the investigation of angiogenesis and lymphangiogenesis at the same time. We recently introduced the rat mesentery culture model as a tool for investigating pericyte-endothelial cell interactions during angiogenesis [42]. We demonstrated that after culture, mesenteric tissues remain intact and viable. And endothelial cells, smooth muscle cells and pericytes remain present in their *in vivo,* pre-cultured location [42]. We also confirmed the presence of lymphatic vessels and that cells remain viable.

The objective of this study was to determine whether the rat mesentery culture model can also be used to study lymphangiogenesis. We show that 1) VEGF-C stimulates both angiogenesis and lymphangiogenesis in the rat mesentery culture model 2) lymphatic sprout extensions maintain their LYVE-1, Prox1, and podoplanin-positive identity and 3) lymphangiogenesis lags angiogenesis similar to the *in vivo* scenario. Our results implicate the rat mesentery culture model as a novel lymphangiogenesis assay, in which you could also investigate angiogenesis at the same time in the same microvascular network scenario. This study serves to inspire new types of experiments aimed at discovering and elucidating the cellular dynamics involved in lymphatic/blood vessel patterning.

Material and Methods

Rat Mesentery Culture Model

All experiments were performed in accordance with the guidelines of the Tulane University Institutional Animal Care and Use Committee. The rat mesentery culture model was used as described previously [42]. Briefly, adult male Wistar rats (Harlan, Indianapolis, IN) were anesthetized and euthanized, and then the mesentery was surgically exposed under aseptic conditions and mesenteric windows were harvested starting from the ileum (Figure 1A). A mesenteric window was defined as the thin, translucent connective tissue between artery/vein pairs that feed the small intestine. Tissues were rinsed in DPBS and cultured at 37°C in 12-well culture plates with serum-free minimum essential media (MEM) supplemented with penicillin/streptomycin (all from Life Technologies, Carlsbad, CA) with or without recombinant human VEGF-C (10–200 ng/ml, R&D Systems, Minneapolis, MN) changed daily for 3 or 5 days (Figure 1A).

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Immunohistochemistry

Cultured tissues were rinsed in PBS, mounted on glass slides, and fixed in methanol at −20°C for 30 minutes. Tissues were washed in PBS + 0.1% saponin (Sigma-Aldritch, St. Louis, MO) three times for 10 minutes each then immunolabeled with primary antibodies and fluorescently labeled secondaries. Blood vessels were detected with a mouse monoclonal biotinylated antibody against PECAM (1:200, BD Pharmingen, San Diego, CA) along with a Cy3-conjugated streptavidin (1:500, Jackson Immunoresearch, West Grove, PA). Lymphatic vessels were detected with either a rabbit polyclonal antibody against LYVE-1 (1:100, AngioBio, Del Mar, CA) along with an Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:100, Jackson Immunoresearch), a mouse monoclonal antibody against Prox1 (1:100, Novus Biologicals, Littleton, CO) along with an Alexa Fluor 594-conjugated goat anti-mouse antibody (1:100, Jackson Immunoresearch), or a mouse monoclonal antibody against podoplanin (1:100, AngioBio) along with an Alexa Fluor 594-conjugated goat anti-mouse antibody (1:100, Jackson Immunoresearch). Proliferating cells were identified by incubating the tissues with BrdU (Sigma-Aldritch) for 2 hours under standard culture conditions. Tissues were then fixed as before and incubated in 6 M HCl for 1 hour to denature the DNA. Tissues were washed and labeled with a mouse monoclonal antibody against BrdU (1:100, Dako, Carpinteria, CA) along with an Alexa Fluor 594-conjugated goat anti-mouse antibody (1:100, Jackson Immunoresearch).

Quantification of Lymphangiogenesis and Angiogenesis

Tissues were randomly selected for quantification of blood capillary sprouts and lymphatic sprout extensions (n=5–6 tissues from 2–4 rats). For the quantification of different VEGF-C concentrations, n=12 tissues from 7 rats for the control group. This was to ensure there was no significant baseline variation between sets of experiments. The blood and lymphatic vascular area of each tissue was imaged at 4x magnification, where vascular area was defined as the area circumscribed by the sum of each individual network within a tissue. The number of lymphatic sprout extensions and blood capillary sprouts were quantified per respective vascular area. Blood and lymphatic vessels were identified by PECAM and LYVE-1 expression, respectively. Blood capillary sprouts were defined as blind-ended blood vessel segments extending from existing vessels. Lymphatic sprout extensions were defined as LYVE-1-positive endothelial cell extensions off a host vessel. They were differentiated from typical lymphatic blind ends, which are the terminal, blunt-ended vessels associated with a functional initial lymphatic network [4,45], based on a diameter and morphology similar to blood capillary sprouts. Filopodia are short pseudopod projections that are typically less than the diameter of the host vessel. In contrast, lymphatic sprout extensions exhibited a length longer than the host diameter. Lymphatic filopodia were quantified from five randomly selected 10x fields of view per tissue (n=6 tissues per group from 3–4 rats) and normalized to the total lymphatic vessel length per image. Lymphatic vessel proliferation was quantified by the number of BrdU+ cells identified within LYVE-1+ vessels normalized to the total lymphatic length from a representative five-by-five 10x mosaic image per tissue (n=4 tissues per group from 2 rats). Images were acquired using an Olympus IX70 inverted microscope with 4x dry, 10x dry, 20x oil, and 60x oil objectives and coupled to a Photometrics CoolSNAP EZ camera. Image analysis and quantification was done using ImageJ 1.45s [36].

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Statistical Analysis

Comparison of lymphatic sprouting between VEGF-C concentrations was analyzed by one-way ANOVA with Tukey pairwise comparison. Lymphatic filopodia and proliferation results were compared by unpaired Student's *t* test. The time course of lymphatic and blood capillary sprouting were analyzed by two-way ANOVA with Tukey pairwise comparison. Results were considered statistically significant when $p < 0.05$. Statistical analysis was performed using SigmaStat ver. 3.5 (Systat Software, San Jose, CA). Values are presented as means ± standard error of the means.

Results

VEGF-C Stimulates Lymphatic Sprout Extension off Intact Initial Lymphatic Networks

Initial lymphatic and blood microvascular networks remained intact in rat mesenteric tissues cultured for 3 and 5 days in culture (Figure 1). Additionally, vascular cells along the intact microvascular networks remained viable [42]. The initial lymphatics, characterized by their lack of smooth muscle cells, along the hierarchy of lymphatic networks were distinguished from blood vessels based on 1) decreased PECAM labeling, 2) increased vessel diameters and 2) positive labeling for LYVE-1, Prox1, and Podoplanin. To assess the growth potential of lymphatic vessels in the model, serum-free media was supplemented with VEGF-C, a known lymphangiogenic growth factor [26]. Treatment with 100 ng/ml VEGF-C resulted in a significant increase in lymphatic sprout extensions after five days (Figure 2). A concentration of 100 ng/ml was chosen based on levels previously reported to stimulate lymphatic endothelial cells and lymphangiogenesis [10,16,27]. The use of 100 ng/ml VEGF-C was also supported by dose response data (Figure 2D). A low concentration of VEGF-C (10 ng/ml) did not result in a significant difference in lymphatic sprout density, while lymphatic sprouting with 200 ng/ml VEGF-C was comparable to 100 ng/ml levels. Lymphatic sprout extensions were defined as long, LYVE-1-positive endothelial cell extensions off a host vessel and were often multicellular (Figures 2C and 3A–C). Lymphatic sprout extensions were also distinguished from normal blind-ended lymphatic vessels by their smaller diameter and lack of the typical blunt and rounded morphology. Importantly, lymphatic sprout extensions were present along the entire hierarchy of the intact networks (Figure 2B and C) and maintained their LYVE-1/Prox-1/Podoplanin-positive phenotype (Figure 3). Evidence for lymphangiogenesis in the rat mesentery culture model was further supported by the presence of LYVE-1-positive filopodia extending from both blind-ended lymphatics (Figure 4B) and along the length of lymphatic vessel segments (Figure 4C). The number of filopodia, which have been previously associated with lymphangiogenesis [5,6], was significantly increased in the presence of 100 ng/ml VEGF-C compared to control (Figure 4D).

Lymphangiogenesis Lags Angiogenesis in VEGF-C Stimulated Microvascular Networks

The presence of intact lymphatic and blood microvascular networks after culture highlights the potential use of the rat mesentery culture mode as an *ex vivo* tool to study the relationships between lymphangiogenesis and angiogenesis. Recently, our laboratory demonstrated that lymphangiogenesis temporally lags angiogenesis in the rat mesentery following *in vivo* inflammatory stimulus [45]. Similar to the *in vivo* scenario,

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lymphangiogenesis lags angiogenesis in VEGF-C stimulated microvascular networks in culture (Figure 5). Treatment with 100 ng/ml VEGF-C resulted in a significant increase in the blood capillary sprouting compared to controls on day 3 (Control: 13.8 ± 1.6 vs. VEGF-C: 22.2 ± 1.8 sprouts per area; $p < 0.05$) (Figure 5A and C). The number of lymphatic sprout extensions per vascular area on day 3 was not changed compared to the respective control group (Figure 5D and F). On day 5, lymphatic sprouting was significantly increased along the hierarchy of intact lymphatic networks compared to controls (Control: 3.6 ± 0.4 vs. VEGF-C: 6.5 ± 0.6 sprouts per area; $p < 0.01$) (Figure 5E and F). The ability to observe lymphangiogenesis and angiogenesis in the controlled tissue environment *ex vivo* enables new types of investigation aimed at identifying and elucidating multi-system relationships. These include temporal relationships, spatial interactions, and common or divergent cellular dynamics. In support of these potential types of studies, we observed that BrdU-positive proliferating nuclei were commonly located along blood vessels versus lymphatic vessels after 5 days in VEGF-C stimulated microvascular networks (Figure 6A). Quantification of the number of proliferating cells along lymphatic vessels showed that there was no significant difference at day 5 as a result of 100 ng/ml VEGF-C treatment (Figure 6B). We also observed the phenomena of physical connections between lymphatic and blood capillaries, defined by PECAM-positive junctional continuity (Figure 7). Quantification of the number of lymphatic/blood connections did not show a significant change as a result of VEGF-C treatment (data not shown).

Discussion

Lymphatic dysfunction is directly linked to inflammation and multiple pathological conditions. The design of molecular based therapies aimed at reversing this dysfunction requires a better understanding of the coordination between lymphatic vessels and blood vessels. Critical questions remain unanswered: Are angiogenesis and lymphangiogenesis related? Is lymphatic function influenced by the growth of nearby blood vessels? Does lymphatic growth influence interstitial fluid balance? Answers to these questions have motivated an emerging area of research focused on lymphatic/blood vessel patterning. We know that the coordination between lymphatic and blood vessels is critical in adult tissues, yet our understanding is limited by the lack of an *ex vivo* model that allows for mechanistic investigation of both lymphatic and blood vessels in the same environment.

Recent examples of lymphatic blood perfusion in transgenic mice have further emphasized the need for such a model. Intravenous injection of either FITC-labeled dextran or BSI-lectin identified lymphatic perfusion in mice lacking SLP-76[1], Rac1 [12], O-glycan [15], or Fiaf [3]. Similar results were documented in Prox1 conditional-mutant mice [18] and Jensen et al. demonstrated that local environmental factors can induce lymphatic blood perfusion [11]. Work from our laboratory discovered that direct lymphatic/blood connections at the capillary level in adult rat mesenteric microvascular networks can be influenced by local growth factor and inflammatory conditions [37,42,45]. These findings combined with the examples from the literature emphasize the need to investigate how and why lymphatic/blood patterning can go wrong in adult tissues.

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In the current study, we establish that lymphangiogenesis and angiogenesis can be induced in the rat mesentery culture model implicating its novelty as a tool for lymphatic/blood patterning studies. Since vessel remodeling occurs within the intact tissue, the model system is self-contained and does not require embedding into a matrix. It is simple and takes advantage of the mesentery thinness. The rat mesentery has been reported to be 20 μm – 40 μm thick [34] and, consequently, enables observation across the hierarchy of intact networks down to a single cell level. Because of this, characteristic chronic *in vivo* experiments using the rat mesentery has proven valuable for identifying novel cellular phenotypes at specific vessel locations during angiogenesis and lymphangiogenesis [2,30,43–45]. Norrby and Franzen showed more than 30 years ago that the rat mesentery could be cultured to examine the proliferation and cytochemical activity of interstitial cell populations during wound healing [35]. More recently, Chatterjee and Gashev used a similar approach to investigate mast cell accumulation along contractile lymphatic vessels in the mesentery [9]. They cultured excised mesentery sections using specially designed tissue chambers to allow imaging of the same vessel during inflammatory treatments. Motivated, in part, by these examples, we have demonstrated that the rat mesentery culture model could also be used to study pericyte-endothelial cell interactions during angiogenesis and lymphatic/blood endothelial cell connections in the intact microvascular networks [42]. The results from the present study demonstrate that this model can also be used to study lymphatic sprouting across the hierarchy of the initial lymphatic networks. VEGF-C addition to the culture media caused an approximate 2-fold increase in lymphatic endothelial extensions. This increase is comparable to the *in vivo* lymphangiogenic responses in the same tissue [6,45]. Lymphangiogenesis in our model is further supported by the observation of branching and multiple cell nuclei along the endothelial cell extensions. The lymphatic sprout and filopodia extensions are consistent with typical lymphangiogenic morphologies [5,6]. We also know that the lymphangiogenic response is not VEGF-C specific. Media supplementation with bFGF causes filopodia extension by 3 days in culture [42]. The addition of serum also causes both filopodia and sprout extensions (data not shown). Additional experiments are needed to determine whether the increase in lymphatic sprout extensions leads to an increase in lymphatic vessel density or whether the new lymphatic segments adopt a more mature, characteristic blunt-ended morphology.

Compared to the lymphatic ring model [7], lymphatic sprouting in the rat mesentery culture model occurs off of initial lymphatic vessels across an intact network and provides the opportunity to also investigate angiogenesis at the same time. To our knowledge the retinal explant assay is the only other *ex vivo* model that might be able to offer this advantage [29,39]. Lymphangiogenesis, however, has yet to be demonstrated in the retinal explant culture assay. Thus, the rat mesentery culture model potentially offers a set of capabilities which are not possible with chronic *in vivo* studies and have not been demonstrated with any other *ex vivo* culture model.

A limitation of the model is the lack of flow. While the lack of hemodynamic forces along blood and lymphatic vessels certainly impacts the physiological relevance, this limitation is shared by most other *ex vivo* and *in vitro* systems. In recent years, microfluidic device platforms have been [8,40]. The advancement of the field will depend on merging of bottom

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up approaches and our top down approach that takes advantages of the multi-cellular/multi-system arrangement of an intact tissue. Unfortunately, the same mesentery tissue in mice is mostly avascular eliminating the potential for using transgenic mice lines [34]. So for now, we recognize that rationale for the use of our rat mesentery culture model, along with the microfluidic based models and more traditional assays, remain question specific.

Still, the ability to investigate the interrelationships between angiogenesis and lymphangiogenesis in the same microvascular network scenario inspires new types of experiments aimed at discovering and elucidating the cellular dynamics involved in lymphatic/blood vessel patterning. Aside from the interests in understanding the coordination between the two systems, future application of the rat mesentery culture model serves to add new insights regarding the plasticity between blood and lymphatic endothelial cells. Work by Lin et al. showed that lysophosphatidic acid can cause lymphatic marker upregulation by HUVECs [25]. Kang et al. demonstrated that lymphatic versus blood endothelial cell identity is controlled by cell fate regulators including Notch, COUP-TFII and Prox1 signaling [19]. The necessity of Prox1 for maintenance of lymphatic identity in culture was also shown via siRNA inhibition *in vitro* by Johnson et al. [18]. Induction of either angiogenesis or lymphangiogenesis from embryoid bodies depending on control of the local chemical environment [14,24] also highlight the common lineage between lymphatic and blood endothelial cells. These examples of lymphatic/blood endothelial cell plasticity and our observation of lymphatic/blood vessel connections in cultured rat mesenteric tissues motivate future studies using our the rat mesentery culture more to determine whether cell plasticity in an intact microvascular network scenario leads to conversion of vessel types.

Molecular players, such as vascular endothelial growth factors and angiopoietins, are involved in angiogenesis and play similar roles in lymphangiogenesis [20]. VEGF-C is known to stimulate both lymphangiogenesis and angiogenesis [6,20,41]. In our study, we also show that this is the case. VEGF-C caused an increase in blood capillary sprouting at day 3 and an increase in lymphatic sprouting at day 5. It should be noted that at day 5, the number of blood capillary sprouts per vascular area was comparable in the VEGF-C versus control groups. The apparent increase in blood vessel sprouts for the control group at day 5 indicates a baseline level of endogenous angiogenesis. Such a response has been associated with the aortic ring model [31–33] and the induction of stimulation from the tissue harvesting method. Given that chronic angiogenesis in the rat mesentery *in vivo* can be stimulated by simply exteriorizing the tissue [47], a sham-like response in our culture system is not surprising. This effect, however, on the time duration for angiogenesis and lymphangiogenesis studies remains to be investigated. Our first characterization of the rat mesentery culture model suggests that tissues can remain viable for at least 7 days [42]. Future experiments, however, are needed to determine how long the tissues can remain intact without cell de-differentiation. Nonetheless, the temporal lag in lymphangiogenesis is consistent with *in vivo* observation by us and others [5,45]. VEGF-C binds to VEGFR-3 along lymphatic endothelial cells and to either VEGFR-3 or VEGFR-2 along blood endothelial cells. We do know that both vessel types positively label for VEGFR-3 in the rat mesentery (data not shown).

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Application of the rat mesentery culture model also offers the potential to elucidate how a specific growth factor influences one growth process versus the other in the same tissue space. VEGF-C stimulation has been reported to increase lymphatic endothelial cell proliferation [6,13,16,22]. Somewhat surprisingly, VEGF-C did not cause proliferation along lymphatic vessels at day 5. This inconsistency with the literature warrants future investigation and might be explained by differences between the *in vivo* and *ex vivo* scenario or the timing of the BrdU pulse labeling. Since the BrdU labeling was performed at after 5 days in our study, any proliferating nuclei at earlier time points would have been missed. Nonetheless, our observation of BrdU positive cells along apparent blood vessels highlights the potential utility of our model to identify differential blood and lymphatic vessel specific responses in the same tissue. In spite of the lack of proliferation along lymphatics, the VEGF-C dose dependent increase in lymphatic cell extensions suggests a preferential effect of VEGF-C on migration. A lymphatic network growth response is supported by 1) the observation of filopodia, which have been previously documented to be a characteristic of lymphangiogenesis, and 2) the association with angiogenesis. Our previous use of antibodies to attenuate capillary sprouting in the rat mesentery culture model also provides rationale for future studies to examine the differential effects of signaling inhibition on lymphatic versus blood vessel growth.

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List of Abbreviations

BrdU	bromodeoxyuridine
bFGF	basic fibroblast growth factor
DPBS	Dulbecco’s phosphate buffered saline
HUVEC	human umbilical vein endothelial cell
LYVE-1	lymphatic vessel endothelial hyaluronan receptor 1
PBS	phosphate buffered saline
PECAM	platelet endothelial cell adhesion molecule
Prox1	prospero homeobox protein 1
MEM	minimum essential media
VEGF-C	vascular endothelial growth factor C
VEGFR-2	vascular endothelial growth factor receptor 2
VEGFR-3	vascular endothelial growth factor receptor 3

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Perspectives

Our study demonstrates that lymphangiogenesis can be induced in the rat mesentery culture model. The ability to investigate both lymphangiogenesis and angiogenesis in an intact microvascular network scenario *ex vivo* offers a potential new tool for future investigations aimed at better understanding the interrelationships between angiogenesis and lymphangiogenesis. This multi-cellular/multi-system test bed might also offer an attractive tool for the development of drug testing or tissue engineering strategies.

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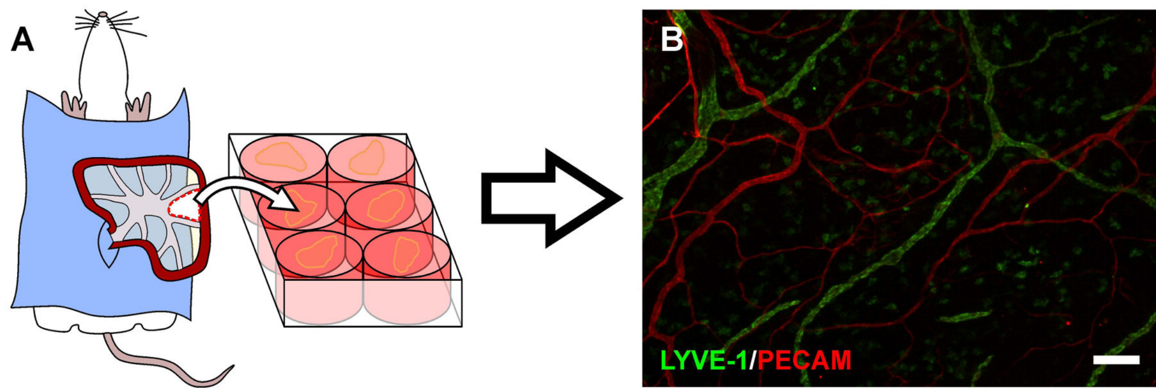

Figure 1.

Rat mesentery culture model. A) Mesenteric tissues are harvested and cultured in multiwell plates. B) After 3 days, blood and lymphatic vessels remain intact. PECAM labeling identified both blood vessels and lymphatic vessels, while LYVE-1 identified only lymphatic vessels, across the hierarchy of their respective networks. Scale bar = 200 μm.

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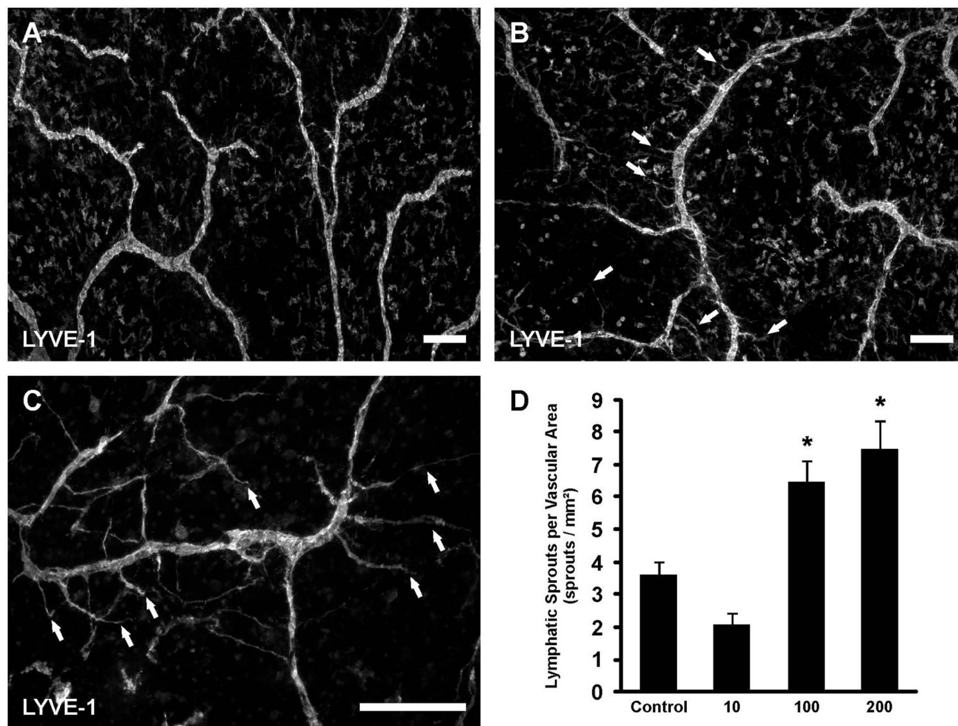

Figure 2.
Lymphatic sprouting in the rat mesentery culture model. LYVE-1 labeling identified lymphatic vessels across the hierarchy of cultured lymphatic networks at 5 days. A) Lymphatic vessels in the control group. B, C) Lymphatic sprouting (arrows) increased in 100 ng/ml VEGF-C stimulated tissues. Lymphatic sprout extensions were characterized by long endothelial cell extensions along the hierarchy of the lymphatic network (C, arrows). D) Quantification of the number of lymphatic sprout extensions per vascular area after treatment with various concentrations (ng/ml) of VEGF-C for 5 days. * represents a significant difference compared to control group. Scale bars = 200 μm.

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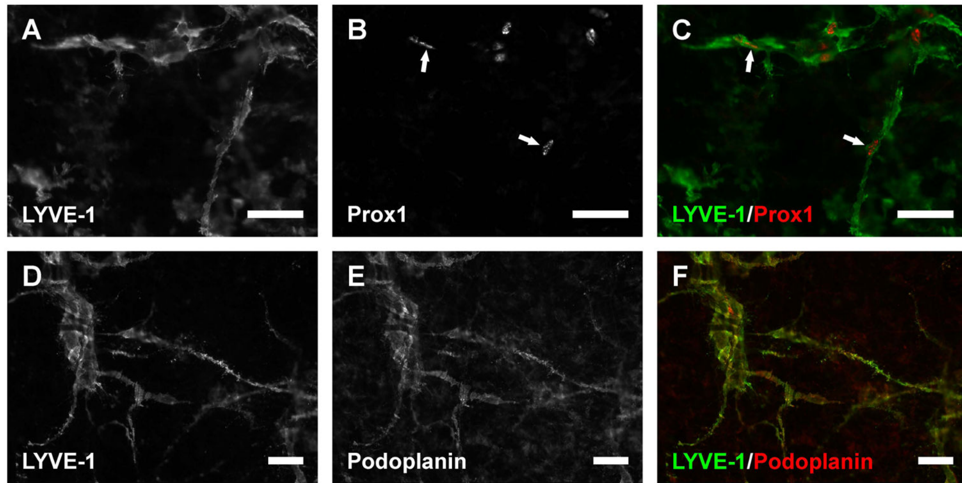

Figure 3.
Examples of lymphatic marker expression along sprout extensions. LYVE-1 expression co-localized with Prox1 (A–C) and podoplanin (D–F). Arrows indicate Prox1-positive nuclei along lymphatic sprout extensions. Scale bars = 50 μm.

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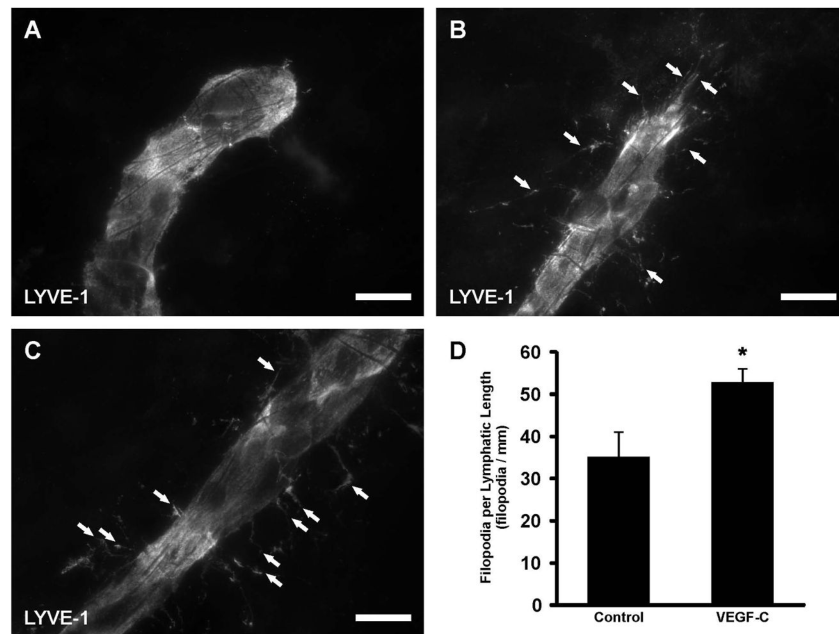

Figure 4.
Examples of LYVE-1-positive filopodia along lymphatic vessels after 5 days in culture. A) Lymphatic blind end without filopodia from control group. B) Lymphatic blind end with typical filopodia after 100 ng/ml VEGF-C treatment. C) Filopodia were also identified along the lengths of lymphatic vessels after 100 ng/ml VEGF-C treatment. D) Lymphatic filopodia were significantly increased in 100 ng/ml VEGF-C treated groups at day 5. * represents a significant difference compared to control group. Scale bars = 20 μm.

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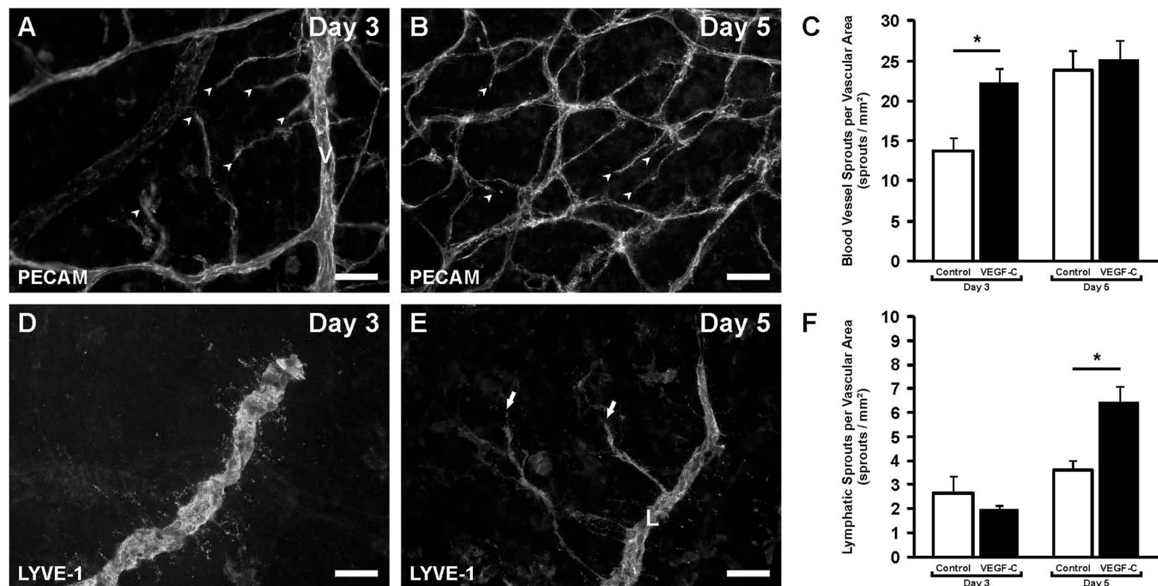

Figure 5.

Lymphangiogenesis lags angiogenesis in the rat mesentery culture model. A–C) Blood capillary sprouting (arrowheads) was significantly increased by day 3 (A) and remained elevated through day 5 (B) during culture with 100 ng/ml VEGF-C. D–F) Lymphatic sprout extensions (arrows) were significantly increased by day 5 (E), but not day 3 (D), during culture with 100 ng/ml VEGF-C. This temporal relationship is consistent with our observations *in vivo* (Sweat et al., 2012). “V”, venule. “L”, lymphatic vessel. * represents a significant difference compared to control group. Scale bars = 50 μm.

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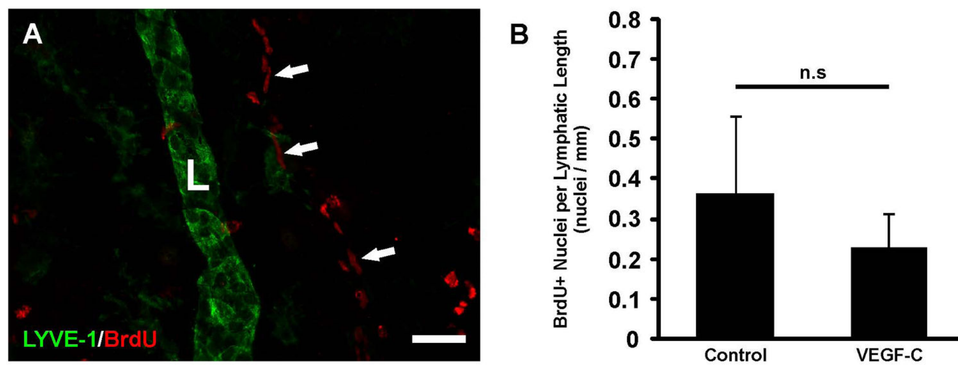

Figure 6.
Proliferation in a VEGF-C stimulated microvascular networks after 5 days. A) LYVE-1 labeling identified lymphatic vessels. BrdU-positive nuclei were commonly located along apparent blood vessels (arrows), but did not co-localize with lymphatic vessels (L). BrdU co-localization along blood vessels was confirmed via subsequent BSI-lectin labeling (data not shown). B) Quantification of BrdU+ cells along lymphatic vessels showed no significant difference in the 100 ng/ml VEGF-C treated group versus control. “L”, lymphatic vessel. “n.s.” represents no significant difference compared to control group. Scale bar = 50 μm.

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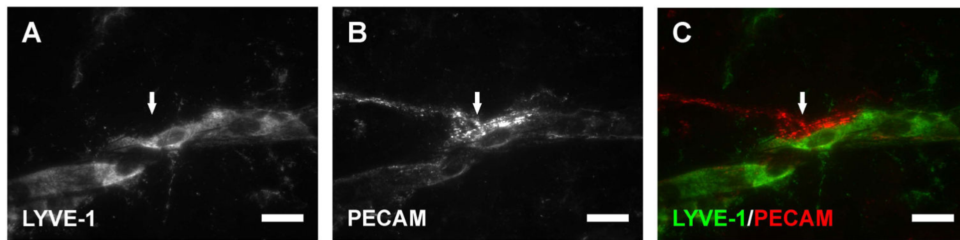

Figure 7.

Example of a lymphatic/blood vessel connection at day 3 in a microvascular network stimulated by 100 ng/ml VEGF-C. LYVE-1 labeling identified lymphatic endothelial cells at PECAM-positive connection sites (arrows). Continuous PECAM junctional labeling by endothelial cells across connection sites has been previously confirmed in sub-micron optical sections using confocal microscopy [37,42,45]. Scale bars = 20 μm.