A New Approach to Visualize and Characterize the Lymphatic Vasculature in Atherosclerosis

PhD Thesis

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1. Introduction

The lymphatic system is a low-pressure vascular system consistent of lymphatic vessels, nodes, and organs such as the spleen and tonsils. Traditionally, its primary functions include dietary lipid absorption, immune cell trafficking, and the transport of interstitial fluid and macromolecules.

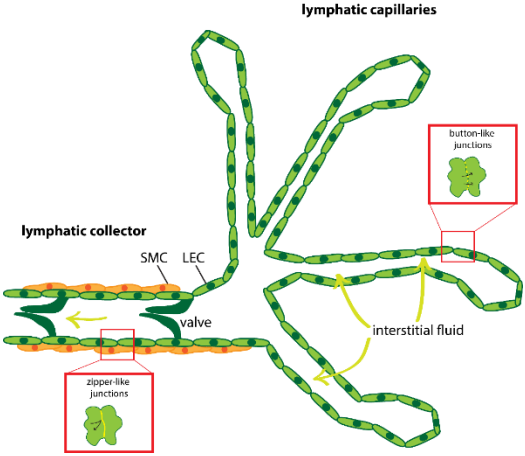

Figure 1: Structure of lymphatic collectors and capillaries. *Blindly ending lymphatic capillaries connected with button-like junctions between the lymphatic endothelial cells (LECs) take up interstitial fluid and macromolecules from the interstitial space. Fluid is transported by contracting lymphatic collectors with the help of smooth muscle cells (SMC) and valves that ensure unidirectional lymph flow.*

Lymphatic vessels play a crucial role in immune responses by facilitating the transport of antigens and immune cells from peripheral tissues to lymph nodes. Lymphatic capillaries, characterized by highly permeable thin walls and button-like junctions, absorb interstitial fluid and transport it as lymph.

During embryonic development, lymphatic vessels originate from the cardinal vein through the expression of Prox1, a key transcription factor. The growth and maturation of lymphatic vessels are regulated by VEGF-C and its receptor VEGFR-3, essential for lymphangiogenesis. Dysfunction in these pathways can lead to lymphedema, highlighting their importance in vascular development.

Lymphatics can be found in nearly every organ of the mammalian body. Besides the traditional functions, lymphatic vessels have been found to have important organ-specific roles, not only in the maintenance of healthy organ function, but also during development and tissue regeneration. Dysfunction in these vessels is linked

to conditions such as lymphedema, inflammatory diseases, tumor progression and other metabolic diseases.

Atherosclerosis is a chronic inflammatory disease of medium and large sized arteries and is characterized by endothelial damage and influx of low-density lipoprotein (LDL) into the intimal space of the arterial wall. LDL levels are regulated via reverse cholesterol transport (RCT), a mechanism that has been linked to lymphatic vessels. The presence of lymphatic vessels in the arterial wall as well as their involvement in lipoprotein metabolism and RCT has led to the question if the lymphatic vasculature is involved in the development of atherosclerosis. Arterial lymphatic vessels have been studied, but thorough characterizations are missing, mainly due to the lack of efficient visualization techniques.

Further research is necessary to understand its precise role in disease progression and to be able to develop potential targeted therapies.

2. Objectives

Lymphatic vessels are present in the arterial wall and participate in reverse cholesterol transport, suggesting a role in the development of atherosclerosis. However, their precise role in this disease remains largely unknown, primarily due to inefficient visualization techniques. During my PhD studies I aimed to:

- Develop an efficient approach to visualize the organ-specific lymphatic vasculature.
- Develop a straightforward, accessible method to quantify organ-specific lymphatic growth without the necessity of highly specialized equipment.
- Characterize the organ-specific lymphatic vasculature of the *Flt4*$^{kd/+}$ mouse model to visualize and quantify the organ-specific lymphatic vasculature.
- Visualize and characterize the lymphatic vasculature of the great vessels under physiological conditions in detail.
- Characterize atherosclerotic plaque development in the *Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$ mouse model, two well-

known atherosclerotic mouse models with focus on sex-dependent differences.

- Visualize and characterize the lymphatic vasculature of the *Ldlr*$^{-/-}$ mouse model in atherosclerosis.

3. Materials and Methods

3.1 Mouse Models

We used 3–5-month-old male and female *C57BL/6* and *Prox1*^{GFP} BAC transgenic lymphatic reporter mice, genotyped by PCR. Additionally, *Flt4*$^{kd/+}$ mice with lymphatic dysfunction and their littermate controls were maintained on an NMRI background. *Flt4*$^{+/+}$ and *Flt4*$^{kd/+}$ mice were crossed with *Prox1*^{GFP} reporters. For atherosclerosis studies, we used 20-week-old *C57BL/6* controls and 23–33-week-old *Ldlr*$^{-/-}$ and *ApoE*$^{-/-}$ mice. All animals were housed under a 12/12 h light/dark cycle with ad libitum food and water, following institutional ethical guidelines.

3.2 Special Diet for Atherosclerosis Development

Atherosclerosis mice received either a control diet (5% fat, 0% cholesterol) or a Western diet (21% fat, 0.21% cholesterol) for 20–30 weeks, starting 21 days post-birth. Equal numbers of mice on control and Western diet were used for comparability.

3.3 Body Weight and Serum Cholesterol Measurements

Body weight was recorded at the end of the diet before mice were sacrificed. Serum cholesterol, triglycerides, HDL, and LDL were measured using an AU480 chemistry analyzer.

3.4 Histology and Staining

Paraffin sections were prepared, and standard H/E, Masson-Trichrome, Alizarin Red S, and LYVE1 immunostaining were performed. Sections were imaged via upright/stereo microscopy.

3.5 In Situ and Whole-Mount Staining of Aortas

Aortas were imaged in situ and stained using Oil Red O or Alizarin Red S for plaque visualization. Tissue processing followed established protocols.

3.6 Tissue Clearing and Whole-Mount Immunostaining

A modified CUBIC protocol that we optimized was used for tissue clearing, with stepwise delipidation,

immunostaining (LYVE1, GFP, αSMA), and refraction index adjustment for imaging.

3.7 Imaging and Quantification

Images were acquired via microscopy and analyzed using NIS-Elements and ImageJ. Atherosclerotic plaques, fibrosis, and lymphatic structures were manually or computationally quantified using AngioTool.

3.8 Statistical Analysis

All figures shown in this work are representative images of the experiments. Data were analyzed using GraphPad Prism and Excel. Normality tested via Shapiro–Wilk, and comparisons made using ANOVA or non-parametric tests. A p-value <0.05 was considered significant.

4. Results

4.1 Visualization of Organ-Specific Lymphatic Growth

4.1.1 Traditional Methods for Lymphatic Visualization

We used lymphatic reporter mice and immunostainings to visualize lymphatic vasculature in various mouse organs. The *Prox1*^{GFP} model allowed observation of superficial lymphatic vessels in the ear skin, small intestine, lungs, and aorta. Immunostainings with anti-LYVE1 identified superficial lymphatics, but these traditional methods did not allow detailed characterization of the lymphatic network due to the opacity of native tissue.

4.1.2 Optimization of the Tissue-Clearing Approach

We improved the CUBIC tissue-clearing protocol by Susaki et al., allowing efficient clearing of multiple mouse organs without specialized equipment. This protocol resulted in high tissue transparency, enhancing visualization of lymphatics in diverse organs, including the heart, reproductive organs, and intestines.

4.1.3 Lymphatic Visualization Using Tissue Clearing

Using our optimized protocol, we successfully visualized lymphatic networks in multiple organs via whole-mount

immunostaining. Lymphatic capillaries in the ear skin, intestinal lacteals, and lung lymphatics were clearly observed. The method also enabled imaging of lymphatic vessels surrounding major arteries, the uterus, testicles, and epididymis. Additionally, we confirmed long-term fluorescence stability in stored samples.

4.1.4 Lymphatic Quantification with AngioTool

We evaluated AngioTool for analyzing lymphatic networks, finding it effective for quantifying lymphatic junctions and endpoints. Manual validation confirmed its accuracy compared to traditional counting methods.

4.1.5 Morphological Changes in *Flt4*$^{kd/+}$ Mice

Using tissue clearing, we examined lymphatic growth in *Flt4*$^{kd/+}$ mice. These mice lacked detectable lymphatics in the skin and exhibited dilated lymphatic vessels in the intestines, lungs, heart, and uterus. The number of lung lymphatics was reduced, and lymphatic networks did not extend fully.

4.1.6 Quantification of Lymphatic Changes in *Flt4*$^{kd/+}$ Mice

Histological and tissue-cleared imaging revealed significant morphological differences in *Flt4*$^{kd/+}$ mice, including fewer but larger lymphatic vessels in the lungs and intestines. Our approach effectively detected changes in lymphatic area, vessel length, and network complexity.

4.2 Lymphatic Vasculature in Atherosclerosis

4.2.1 Lymphatic Presence in the Great Vessels

Immunostaining of arterial sections from wild-type mice revealed lymphatic vessels in the thoracic and abdominal aorta, but not in the aortic arch. Quantification confirmed variations in lymphatic density across different aortic regions.

4.2.2 Effects of Western Diet on Body Weight and Lipid Levels

Ldlr$^{-/-}$ and *ApoE*$^{-/-}$ mice on a Western diet gained significant weight, with males exhibiting greater increases. Cholesterol and LDL levels were markedly elevated in both male and female mice on the diet.

4.2.3 Sex Differences in Atherosclerotic Plaque Formation

Plaque development in the aortic arch was observed after 16 weeks on a Western diet, with significant accumulation by 20 weeks. Cross-sectional analyses showed larger plaques in females than males.

4.2.4 Plaque Calcification and Fibrosis

Lipid accumulation and fibrosis were more pronounced in Western diet-fed mice. Female mice exhibited greater plaque calcification than males, confirmed by Alizarin Red S staining.

4.2.5 Lymphatic Changes in Atherosclerosis

Immunostaining of atherosclerotic arteries showed no significant differences in lymphatic density or morphology between control and Western diet-fed mice. Only female mice on Western diet showed enlarged lymphatic vessels in the abdominal aorta, but lymphatic vessels remained mainly unaltered in atherosclerosis.

6. Discussion

We developed an effective methodology for visualizing, analyzing, and quantifying organ-specific lymphatic vessel growth. Traditional visualization methods have great limitations due to tissue opacity, but our modified CUBIC-based tissue-clearing protocol overcomes these, enabling high-quality imaging without shrinkage or damage. It is compatible with various fluorescent markers and allows long-term sample preservation. Unlike other protocols, ours is accessible, avoiding the need for specialized equipment.

Using this approach, we characterized lymphatic vasculature in a model of lymphatic dysfunction, confirming previously reported abnormalities while identifying novel morphological changes in multiple organs. Quantification of tissue-cleared samples provided a more comprehensive assessment than traditional sectioning, revealing significant structural alterations indicative of impaired lymphatic function.

Additionally, we examined lymphatic vasculature in atherosclerosis and identified sex-specific differences.

Female mice exhibited increased arterial plaque calcification and enhanced peripheral lymphatic function, suggesting potential sex-related alterations in lymphatic activity. While arterial lymphatic vessels remained largely unchanged, moderate dilation in specific regions hinted at their role in cholesterol clearance. Our findings highlight the importance of considering distinct arterial segments when investigating vascular disease.

Overall, this study underscores the utility of our tissue-clearing protocol for lymphatic vessel analysis, revealing novel insights into organ-specific lymphatics in health and disease. The observed sex-dependent differences in atherosclerosis suggest potential lymphatic contributions to disease progression, warranting further investigation into their functional role.

7. Conclusion

In this study we characterized and quantified organ-specific lymphatic growth with a focus on sex-dependent differences in atherosclerosis, utilizing an optimized tissue-clearing protocol.

- Our optimized tissue-clearing technique provides a straightforward method to visualize and quantify organ-specific lymphatic growth, all without the need for specialized equipment, software, or prior expertise, thus making tissue-clearing available for researchers of all backgrounds.
- Our technique enables the visualization and quantification of morphological alterations in the lymphatic vasculature, making it an efficient tool to visualize and quantify lymphatic disorders.
- In our investigation, we observed dilated and diminished lymphatic structures in the lungs, uterus, and heart of *Flt4*$^{kd/+}$ mice, expanding upon previous findings limited to the skin.
- Throughout our investigation, we found that lymphatic vessels are distributed unevenly within the arterial tree, with sparse presence in the aortic

arch and abundance in abdominal and femoral arteries.

- In our study, we observed that female mice tend to develop larger plaques with significantly larger calcifications compared to males.
- We also noted that the lymphatic vasculature of large vessels remains mostly unaffected by atherosclerosis, except for dilation in the abdominal aorta of female mice on a Western diet.
- Furthermore, mice on a Western diet exhibited enlarged popliteal lymph nodes, particularly pronounced in females.
- Lastly, females showed signs of increased peripheral lymphatic function compared to males on a Western diet.

8. Bibliography of the candidate's publications

8.1 Publications included in this dissertation

I Christ C, Jakus Z.
Visualization of Organ-Specific Lymphatic Growth: An Efficient Approach to Labeling Molecular Markers in Cleared Tissues. Int J Mol Sci. 2023 Mar 7;24(6):5075. doi: 10.3390/ijms24065075. PMID: 36982150.
Impact Factor: 4.9

II Christ C, Ocskay Z, Kovács G, Jakus Z.
Characterization of Atherosclerotic Mice Reveals a Sex-Dependent Susceptibility to Plaque Calcification but No Major Changes in the Lymphatics in the Arterial Wall. Int J Mol Sci. 2024 Apr 5;25(7):4046. doi: 10.3390/ijms25074046. PMID: 38612867.
Impact Factor: 4.9

8.2 Publications not included in this dissertation

III Ocskay Z, Bálint L, Christ C, Kahn ML, Jakus Z.
CCBE1 regulates the development and prevents the age-dependent regression of meningeal lymphatics. Biomed

Pharmacother. 2024 Jan;170:116032. doi: 10.1016/j.biopha.2023.116032. PMID: 38141283.
Impact Factor: 6.9

IV Tauber P, Aichinger B, Christ C, Stindl J, Rhayem Y, Beuschlein F, Warth R, Bandulik S. Cellular Pathophysiology of an Adrenal Adenoma-Associated Mutant of the Plasma Membrane Ca(2+)-ATPase ATP2B3. Endocrinology. 2016 Jun;157(6):2489-99. doi: 10.1210/en.2015-2029. PMID: 27035656.
Impact Factor: 4.3