Characterization of the Organ-specific Roles of Lymphangiogenic Mechanisms in the Meninges and the Placenta

László Bálint

Doctoral theses

Semmelweis University
Doctoral School of Molecular Medicine

Supervisor:
Zoltán Jakus, MD, PhD., Associate Professor

Reviewers:
Zoltán Wiener, PhD., Associate Professor
Ferenc Domoki, MD, PhD., Associate Professor

Chair of the Final Exam Committee:
György Nagy, MD, DSc., Professor

Final Exam Committee Members:
Zsuzsanna Tóth, MD, PhD., Senior Research Fellow
Mihály Józsi, DSc., Professor

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Introduction

The lymphatic system is a blind-ended, linear vessel system that is distinguished from the cardiac circulatory system in its structure, function, and development.

While some functions of the lymphatic system, such as their role in draining interstitial fluid, the transportation of macromolecules and immune cells, and dietary lipid uptake have been known for a long time, numerous novel organ-specific roles of the lymphatic vessels have been described recently. These include the role of the lymphatic system in the reverse cholesterol transport, the systemic blood pressure homeostasis, and the preparation of the lung for the first inflations at birth.

In addition, our understanding of the mechanisms determining the developmental program and maturation of the lymphatic vessels evolves constantly. The initial lymphatic vessels bud from the cardinal vein between the 6^{th} and 7^{th} week of the 40-weeks-long pregnancy in humans and approximately on the 9^{th} embryonic day of the 21-days-long pregnancy in mice, which is considered as the first step of the developmental program of the lymphatic system. Further development and maturation of the lymphatic vessels take place in an organ-specific time and manner. Importantly, the Vascular endothelial growth factor receptor 3 (VEGFR-3)-mediated intracellular pathway plays a central role in the lymphatic developmental program.

Although the lymphatic system is separated from the blood circulation, it has been observed that in absence of specific proteins, such as Podoplanin (PDPN), C-type lectin-like receptor 2 (CLEC-2), Spleen tyrosine kinase (SYK) and Phospholipase C γ2 (PLCγ2), lymphatic vessels become filled with blood. It has been unveiled recently that these proteins are members of a signaling pathway that leads to a lymphatic endothelial cell-induced

aggregation of platelets. Novel findings suggest that this pathway along with the lymphovenous valve and lymphatic valves co-operates to the development and maintenance of the separation of the two vessel systems of the body. Impairment of this pathway causes a retrograde blood flow into the lymphatic vessels that results in impaired lymph flow. It has been suggested by previous *in vitro* experimental results that the lymph flow may play a role in the developmental program of the lymphatic vessels. Mouse strains in which this molecular pathway is impaired may be excellent models for the *in vivo* examination of the role of lymph flow.

In a recent study it was demonstrated that while the primary mesenteric lymphatic vessels form normally in CLEC-2-deficient mice with impaired lymph flow, organ-specific maturation of the mesenteric lymphatic vessels is impaired in these embryos. Moreover, the organ-specific roles of the lymph flow have been also described in the lungs using this model.

According to the classical view, the central nervous system lacks lymphatic vessels. Although sporadic studies suggested that lymphatic vessels may be present in the meninges, these findings were not able to change this dogma. However, in 2015, lymphatic vessels have been demonstrated in mouse meninges, parallel to the venous sinuses and arteries in the dura. Since then, presence of the meningeal lymphatic structures was also confirmed in humans. These results were sufficient to alter the formerly well-accepted paradigm indicating the lack of a classical lymphatic draining system of the central nervous system (CNS). The discovery of the meningeal lymphatic vessels raised the question regarding the function of these lymphatic vessels. Results of several research groups indicate that the meningeal lymphatic vessels are involved in the drainage of macromolecules from the CNS. Other investigators, however, could not confirm these findings in their experiments. At this point it is not clear whether the

meningeal lymphatic vessels contribute to the drainage of the macromolecules from the CNS. Recent studies suggest that the meningeal lymphatics may be involved in the pathomechanism of diseases affecting the CNS, such as Alzheimer's Disease, multiple sclerosis, or autoimmune encephalitis.

It has been demonstrated recently that the meningeal lymphatic vessels develop in the postnatal period and the VEGFR-3 signaling pathway plays a critical role in their organ-specific developmental program. However, other possible regulators of the development of the meningeal lymphatics remain unclear. Defining the function and mechanisms regulating the organ-specific developmental program of the meningeal lymphatic vessels may lead to a better understanding of their physiological and pathological roles.

In parallel with the discovery of the meningeal lymphatics, it has been demonstrated that the endothelia of some unique vessels possess hybrid expression patterns that combine molecular markers specific for lymphatic endothelial cells and blood endothelial cells. Endothelial cells of the Schlemm's canal and the ascending *vasa recta* have been identified to bear both blood and lymphatic-specific markers. In addition, the endothelial cells of the spiral arteries of the placenta were proposed to express lymphatic molecular markers and it is presumed that the VEGFR-3 pathway may play a role in their remodeling.

Spiral arteries undergo a remodeling process during early to mid-gestation to secure the evolving nutritional and oxygenation needs of the growing fetus. In humans, poor spiral artery remodeling may be associated with preeclampsia. However, underlying mechanisms defining preeclampsia remain poorly understood. Therefore, there is a lack of diagnostic biomarkers that are eligible for prediction of the potential risk of preeclampsia. Defining the role of the VEGFR-3 molecular pathway in the spiral artery remodeling

may lead to a better understanding of the underlying mechanisms leading to preeclampsia and may promote the identification of its potential biomarkers and possible therapeutic targets.

Objectives

In our experiments we aimed at characterizing the organ-specific roles of lymphangiogenic mechanisms in the meninges and in the spiral arteries of the placenta.

We investigated:

1. The role of the meningeal lymphatic vessels in the uptake and transport of macromolecules from the CNS
2. The developmental program of the meningeal lymphatics
3. The lymph flow-dependence of the meningeal lymphatic vessels developmental program
4. The role of the VEGFR-3 molecular pathway in the spiral artery remodeling

Methods

Experimental animals: To investigate the function and development of the meningeal lymphatics, lymphatic reporter (*Prox1*^{GFP}, *Flt4*^{YFP}), and PLCγ2-deficient mice were used in addition to wild type mice. These animals were studied at different timepoints during the postnatal period. To characterize the role of VEGFR-3 in the spiral artery remodeling, *Flt4*$^{kd/+}$ mice were used, in which the tyrosine-kinase activity of the receptor is impaired due to a point mutation present in the Colony stimulating factor 1 (FMS)-related receptor tyrosine kinase 4 (*Flt4)* gene that encodes VEGFR-3.

Histology procedures and immunostaining of histology slides: Tissue samples were embedded in paraffin after fixation and dehydration. Tissue sections were then processed for hematoxylin-eosin and fluorescent immunohistochemistry stainings.

Investigation of the meningeal lymphatic structures: Dissected mouse meninges were fixed and processed for whole-mount fluorescent immunostaining protocol for visualization of the meningeal lymphatic vessels.

Monitoring the lymphatic function: To monitor lymphatic function, we injected fluorescently labeled macromolecules or administered fluorescently labeled lipids to the experimental mice. Uptake and transport of labeled macromolecules and lipids was assessed by fluorescent stereomicroscopy.

Quantitative characterization of the phenotype in PLCγ2-deficient mice: To characterize the gut lymphatic phenotype of PLCγ2-deficient and littermate control mice, a clinical score system was used on a 0–4 scale. To monitor the presence of blood in the regional lymph nodes, a score system was used on a 0–4 scale. Structure of the meningeal lymphatic vessels was

assessed using a clinical score system on a 0–15 scale and by the measurement of the total length of the meningeal lymphatic vessels.

Monitoring the spiral artery remodeling: To assess spiral artery remodeling in mouse placentas, hematoxylin-eosin and fluorescent immunohistochemistry stainined histological sections were used.

Preeclampsia phenotype characterization: To investigate the occurrence of symptoms of human preeclampsia in $Flt4^{kd/+}$ females, weight of embryos, placentas and maternal kidneys was determined in addition to measurement of urinary protein concentrations and systolic blood pressure of late-gestation females.

Results

First, we visualized the expression pattern of lymphatic markers in the meningeal compartment. Lymphatic vessels carrying lymphatic markers Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1), Prospero homeobox protein 1 (PROX-1), VEGFR-3, PDPN and pan-endothelial marker Platelet endothelial cell adhesion molecule (PECAM) were detected along the transverse and sagittal sinuses in young adult mice.

To investigate the role of meningeal lymphatics in the uptake and transport of the macromolecules from the CNS, fluorescently labeled macromolecules (70 kDa and 40 kDa molecular weight rhodamine dextran) were injected into the brain parenchyma and into the *cisterna magna* of young adult lymphatic reporter mice. Minimal drainage of the fluorescent tracers was detected into the superficial cervical lymph nodes. In contrast, the transport of the labeled macromolecules was robust to the deep cervical lymph nodes. In addition, fluorescently labeled macromolecules were observed in the meningeal lymphatic vessels after macromolecule injection into the CNS. These experimental results suggest that the meningeal lymphatic vessels contribute to the uptake and transport of macromolecules from the CNS.

Next, we characterized the developmental program of the meningeal lymphatic vessels. While intact meningeal lymphatic vessels were detected in 21 to 32-days-old young adult mice, we observed immature lymphatic structures in the meninges of newborn mice. In parallel with these results, no macromolecule accumulation was detected in the deep cervical lymph nodes after injection into the brain parenchyma of newborn mice. An increasing macromolecule drainage was observed into the deep cervical lymph nodes during the period in which the developmental program of the meningeal

lymphatic vessels occurs. These data indicate that meningeal lymphatic vessels develop postnatally, which process coincides with the increase of the drainage of macromolecules from the CNS to the deep cervical lymph nodes.

In our further experiments we aimed to characterize the role of the lymph flow as a possible regulator of the developmental program of meningeal lymphatics. Blood-filled lymphatic vessels were detected in the small intestine of late-gestational PLCγ2-deficient embryos similarly to those observed in embryos lacking CLEC-2. Moreover, an insufficient maturation of the mesenteric lymphatic vessels was detected in these embryos. These results indicate an impairment of the separation of the blood and lymphatic vessels in mice lacking PLCγ2 that results in defective maturation of the mesenteric lymphatic vessels. These findings suggest that the lymph flow may be reduced in PLCγ2-deficient embryos similarly to the CLEC2-deficient model. Impaired separation of the blood and lymphatic vessels was also observed in young adult PLCγ2-deficient mice. At this point, *Plcγ2*$^{-/-}$ mice bearing severe and less severe (mild) gut phenotype were detected.

To investigate lymphatic function in PLCγ2-deficient mice, fluorescently labeled lipids were administrated to young adult mice. We observed no lipid uptake and transport in the small intestine of *Plcγ2*$^{-/-}$ mice bearing severe gut phenotype. In *Plcγ2*$^{-/-}$ mice bearing mild gut phenotype, a reduced lipid uptake and transport was detected compared to sibling control animals. Similarly, in *Plcγ2*$^{-/-}$ mice with severe gut phenotype a greatly impaired lymphatic function was detected after injection of fluorescently labeled macromolecules into the hind paws of young adult animals. Lymphatic function was found to be less reduced in *Plcγ2*$^{-/-}$ mice with mild gut phenotype. Assessment of drainage of labeled macromolecules to the deep cervical lymph nodes from the CNS after intraparenchymal or into the *cisterna magna* injection revealed a greatly reduced macromolecule

transport in *Plcγ2*$^{-/-}$ mice with severe gut phenotype. A less reduced drainage of macromolecules was detected in *Plcγ2*$^{-/-}$ mice with mild gut phenotype. These findings indicate an impaired lymphatic function and lymph flow in *Plcγ2*$^{-/-}$ mice. Moreover, the reduction of the lymphatic function and lymph flow in PLCγ2-deficient mice correlates with the gut phenotype of the animals. These findings support that the PLCγ2-deficient genetic model is ideal for the *in vivo* investigation of the role of lymph flow in the organ-specific developmental program of the meningeal lymphatics.

Next, we characterized the morphology of the meningeal lymphatic vessels in *Plcγ2*$^{-/-}$ mice. Impaired lymphatic structures were detected in the meninges of young adult PLCγ2-deficient mice. Structural impairment of the meningeal lymphatics was more explicit in *Plcγ2*$^{-/-}$ mice with severe gut phenotype than in *Plcγ2*$^{-/-}$ mice with mild gut phenotype, as confirmed by a clinical scoring system regarding the structural malformations in the meningeal lymphatic system and also by measuring the total length of the meningeal lymphatic vessels. These results indicate a correlation between the impaired maturation process of the meningeal lymphatics and the severity of the altered lymphatic function and gut phenotype in PLCγ2-deficient mice.

Thereafter, the uptake of macromolecules into the meningeal lymphatic vessels was characterized in young adult *Plcγ2*$^{-/-}$ mice after intraparenchymal and into *cisterna magna* injection of the tracer. Macromolecule uptake was greatly impaired in *Plcγ2*$^{-/-}$ mice with severe gut phenotype, and less reduced in *Plcγ2*$^{-/-}$ mice with mild gut phenotype. These results indicate that lymphatic function is reduced in PLCγ2-deficient mice resulting in an impaired maturation of the meningeal lymphatic vessels, reduced macromolecule uptake and transport from the CNS, which alterations correlate with the gut phenotype observed in the animals.

In our further experiments, we investigated the possible role of the VEGFR-3 lymphangiogenic pathway in the spiral artery remodeling. Results of our collaborating partners revealed that murine spiral arteries acquire expression of PROX-1 and VEGFR-3 lymphatic markers during their remodeling.

We found that spiral arteries of placentas derived from *Flt4*$^{kd/+}$ females retain significantly more smooth muscle coverage and fail to undergo the typical increase in luminal area, which are characteristic of spiral artery remodeling and observed in wild type mice. In addition, reduced level of ERK phosphorylation was observed in the epithelium of spiral arteries of placentas derived from *Flt4*$^{kd/+}$ females compared to those in wild type mice. These results indicate an impairment of the intracellular signaling of the VEGFR-3 in the spiral arteries of *Flt4*$^{kd/+}$ females, that results in spiral artery remodeling defects.

We next sought to determine if the *Flt4*$^{kd/+}$ mice exhibit symptoms of preeclampsia during pregnancy. We observed significant fetal growth restriction and increased placental weights at late gestation in *Flt4*$^{kd/+}$ mice with insufficient spiral artery remodeling, that may imply fetal distress. We found no significant differences in systolic blood pressure, urinary protein concentrations and maternal kidney weights and renal structure between *Flt4*$^{kd/+}$ and sibling control mice. These results indicate that *Flt4*$^{kd/+}$ mice exhibit pregnancy deficits compatible with insufficient spiral artery remodeling.

Conclusions

After demonstrating the presence of lymphatic vessels in the meninges we characterized their role in the uptake and transport of macromolecules from the CNS. Our results indicate that meningeal lymphatic structures are involved in the uptake and transport of macromolecules from the CNS into the deep cervical lymph nodes.

We observed that the meningeal lymphatics develop during the postnatal period which process involves the maturation of the vessels. Formation of mature meningeal lymphatic vessels coincides with the increase in macromolecule drainage from the CNS.

Our results revealed that the lymph flow and lymphatic function is impaired in PLCγ2-deficient mice representing a blood-filled lymphatic phenotype. The severity of the lymphatic function and lymph flow reduction correlates with the gut phenotype of the animals. These findings indicate that the PLCγ2-deficient mouse strain is an excellent model for the *in vivo* examination of the role of lymph flow. Importantly, the postnatal maturation of meningeal lymphatics is also affected in PLCγ2-deficient mice with reduced lymph flow, which results in an impaired macromolecule uptake and transport from the CNS. Collectively, the lymph flow plays an important role in the organ-specific maturation of the meningeal lymphatic vessels.

In our further experiments we found that the VEGFR-3 lymphangiogenic pathway is essential for the spiral artery remodeling. Impairment of the intracellular signaling of VEGFR-3 results in spiral artery remodeling defects in $Flt4^{kd/+}$ females, accompanied by a phenotype that is comparable to the human preeclampsia.

Defining the organ-specific roles of lymphangiogenic mechanisms in physiological and pathological processes may lead to a better understanding

of yet incurable diseases and identification of potential therapeutic targets and biomarkers. By revealing the lymph flow-dependence of the development and function of meningeal lymphatics, our results may lead to a better understanding of the organ-specific regulatory mechanism of the developmental program of the meningeal lymphatic vessels, and physiological and pathological roles of these structures. Moreover, our findings may provide a possible therapeutic target for neurological disesases such as Alzheimer’s disease or multiple sclerosis. Our further results suggest that the impairment of the VEGFR-3 pathway may be involved in the pathophysiology of preeclampsia and that the VEGFR-3 may be a potential biomarker and therapeutic target of preeclampsia.

List of Publications

Publications Related to the Thesis

I. ***L. Bálint****, Zs. Ocskay, BA. Deák, P. Aradi, Z. Jakus*

Lymph Flow Induces the Postnatal Formation of Mature and Functional Meningeal Lymphatic Vessels.

Front Immunol. 2020 Jan 14;10:3043. doi: 10.3389/fimmu.2019.03043.

IF: 5,085

II. *JB. Pawlak,* ***L. Bálint****, L. Lim, W. Ma, RB. Davis, Z. Benyó, MJ. Soares, G. Oliver, ML. Kahn, Z. Jakus, KM. Caron*

Lymphatic mimicry in maternal endothelial cells promotes placental spiral artery remodeling.

J Clin Invest 2019;129(11):4912-4921. doi: 10.1172/JCI120446.

IF: 11,864