IDENTIFYING ORGAN-SPECIFIC MECHANISMS TO STIMULATE LYMPHATIC GROWTH AND FUNCTION

PhD Thesis

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1. Introduction

Novel roles of the lymphatic system were identified lately and made us realize that lymphatic vessels vary in an organ specific manner. In this study, we wanted to identify the organ specific functions of the lymphatics in the skin in lymphedema and in the lungs in respiratory adaptation of the newborn.

Lack or dysfunction of the lymphatics in the skin leads to secondary lymphedema. There is no definitive treatment for lymphedema, only symptomatic relief.
VEGFC, and its receptor VEGFR3 together are the most important signaling route for lymphatic growth and maintenance.
There were several attempts to administer VEGFC as a therapeutic, as protein or in virus vector-based systems. All of these methods have several limitations and side effects.
A novel *in vivo* protein expression platform arose lately, the so-called mRNA-LNP system. It was successfully used to develop vaccines during the recent COVID-19 pandemic.

Lately, new organ specific functions of the lymphatics were identified in the developing lung.
Newborn mice look cyanotic, die shortly after birth in respiratory failure in mouse models with disturbed lymphangiogenic factors.

2. Objectives

To understand better the organ-specific physiological and pathophysiological roles of the lymphatics in the skin and in the developing lung, which can potentially lead us to better therapeutic approaches against secondary lymphedema in adult patients and respiratory failure in newborns.

To examine organ-specific function of lymphatics in the skin, we wanted to develop a novel nucleoside-modified VEGFC mRNA platform (VEGFC mRNA-LNP) to induce organ-specific lymphatic growth *in vivo* including the following steps:

- development and production of the VEGFC mRNA-LNP platform;
- characterization of the VEGFC mRNA-LNP system *in vitro;*
- characterization of the VEGFC mRNA-LNP system *in vivo;*
- testing the VEGFC mRNA-LNP complexes in an experimental lymphedema mouse model *in vivo.*

To better understand physiologic and pathophysiologic roles of the lymphatics in the developing lung we aimed the followings:

- setting up a mouse model, in which FBMs are impaired during late gestation embryonic development;
- characterizing the phenotype of $Clp1^{K/K}$ newborn mice;
- setting up an ultrasound-based system to monitor FBMs;
- monitoring the FBMs in $Clp1^{K/K}$ and control embryos during development *in utero.*

3. Methods

Dysfunction of lymphatics in the skin

Poly(C) and VEGFC mRNA-LNP complexes were first produced together with Norbert Pardi and Drew Weissman from UPENN after optimalization and nucleoside modification of the mRNAs. LNP incorporation was made by Acuitas therapeutics Inc.

Then Poly(C) and VEGFC mRNA-LNPs were injected locally via various routes (intradermally into ear and paw, intraperitoneally into diaphragm, intratracheally into lung, intramuscularly into gasctrocnemic muscle).

Wild-type mice (C57Bl6/J), lymphatic vessel reporter mice (*Prox1*^{GFP}), secondary lymphedema mouse model (*Flt4-CreER*^{T2}*; iDTR*$^{fl/f}$) animals were used in our experiments in accordance with ethical documents of Hungary and the Semmelweis University.

VEGFC protein expression analysis was performed with Western Blot and ELISA (anti-VEGFC ab).

Stereo microscopy or paraffin-based histology (H&E or fluorescent immunohistochemistry – first abs: anti-LYVE1, anti-Podoplanin, anti-vWF, anti-CD31 –, and proliferation assay with EdU) was performed after terminalization of the animals.

Flow cytometry was performed to determine possible immune cell infiltration. (First abs: anti-LYVE1, anti-CD45, anti-Ly6G/C, anti-CD206, anti-CD3, anti-B220, anti-CD11b)

Functionality of the lymphatics were measured after injection of fluorescent labeled Rhodamine-Dextran macromolecule into ears or paws of mice.

Lymphedema induction was performed in secondary lymphedema mouse model animals with Tamoxifen treatment intraperitoneally and PBS or DT injection intradermally into paws afterwards.
Isofluran was used to anesthetize the mice.
Measurement of paws was performed by caliper.
NIS-Elements program was used for quantification and Graphpad Prism for statistical analysis and visualization of data.

Dysfunction of lymphatics in the developing lung

Timed pregnancies were set up of $Clp1^{K/+}$ heterozygous animals to produce $Clp1^{K/K}$ and control embryos and newborns.
Cesarean section of newborns was performed at E19.5 gestational days.
In other experiments Isofluran anesthesia of mother animals took place at E18.5 gestational days before section of the abdomen and externalization of the uterus.
Then, embryos of pregnant mice were scanned by using a micro-ultrasound imaging unit (Visualsonics, Vevo 3100 imaging system, B-mode) equipped with an ultrahigh frequency MX400 linear-transducer (30 MHz, 55 frames per second) at E18.5 gestational days.
Tissue samples from embryos were collected for genotyping after scanning.
Genotyping was performed using PCR method.

4. Results

Dysfunction of lymphatics in the skin

VEGFC Protein Is Present in the Supernatant of HEK293T Cells After VEGFC mRNA-LNP Transfection

First, murine VEGFC-encoding 1-methylpseudouridine-containing mRNAs were designed, synthetized, purified, and encapsulated into LNPs in cooperation with Dr. Norbert Pardi from the University of Pennsylvania and Acuitas Therapeutics in Canada.
For the first analysis VEGFC mRNA-LNPs were tested in an *in vitro* experiment. 1 µg of Poly(C) RNA-LNPs or VEGFC mRNA-LNPs were added to the medium of HEK293T (human embryonic kidney 293T) cells. 8 hours, 1, 4, 8 days after the treatment the supernatant of the cells was harvested. VEGFC protein were present and detectable with Western Blot analysis 4 and 8 days after the transfection.

VEGFC Protein Is Expressed *in vivo* Locally After VEGFC mRNA-LNP Administration

Then, *in vivo* effects of VEGFC mRNA-LNP injection were examined. After VEGFC mRNA-LNP injection into one ear of a wild type C57BL/6 (C57 black 6) mice, higher levels of VEGFC protein were measured in the interstitial fluid of the ear compared to the contralateral side injected with Poly(C) (polycytidylic acid) RNA-LNP as control. The elevated VEGFC protein level was present and significant on 1, 5, 10 and 15 days after the injection. On the twentieth day minimal elevation was present but was not significantly higher.

Organ-Specific Lymphatic Growth Is Present After VEGFC mRNA-LNP Injection

Secondly, lymphatic morphology was assessed. One ear of *Prox1*^{GFP} mice was injected with VEGFC mRNA-LNP complexes and the other ear was treated with Poly(C) RNA-LNP as control. Lymphatic endothelial cells of *Prox1*^{GFP} mice express GFP (green fluorescent protein) so it is an excellent tool to assess lymphatic morphology. LYVE1 (lymphatic vessel endothelial hyaluronan receptor 1) is an excellent marker for staining lymphatic endothelial cells.
22 days and 60 days after VEGFC mRNA-LNP administration, significant lymphatic growth was detected in ears. Quantification of the data showed significant increase in the length of the lymphatic network, the average diameter of lymphatic vessels and the number of branching points after 5,

12, 17, 22, 35 and even after 60 days. We also detected a dose dependent lymphatic growing effect. Additionally, Significant increase of all three morphology parameters were observed when injected with as low as 0.04 µg.

We also investigated the effect of VEGFC mRNA-LNP on lymphatics in other organs to show the versatility of this platform. We observed increased lymphatic growth in the diaphragm after intraperitoneal, in the lung after intratracheal, and in the gastrocnemius muscle after intramuscular injection of the VEGFC mRNA-LNP compared to control solution. Quantification of histology slides stained with LYVE1 and Podoplanin lymphatic specific markers showed significant increase in the number of lymphatic vessels.

We performed the so called EdU proliferation assay. EdU (5-ethynyl-2′-deoxyuridine) is a thymidine analogue so it incorporates into DNA of proliferating cells. EdU was administered 5 days after Poly(C) or VEGFC mRNA-LNP treatment of animals, then they were terminated 24 hours thereafter. EdU+ and LYVE1+ proliferating lymphatic endothelial cells and the mitotic index were quantified and calculated from immunohistochemistry images of paraffin-based sections. The number and percentage of EdU positive nuclei of lymphatic endothelial cells were increased in ears injected with 1 µg of VEGFC mRNA-LNP compared to the Poly(C) mRNA-LNP injected ones shown by fluorescent and confocal microscopy.

No Adverse Effect Was Observed After VEGFC mRNA-LNP Injection

To assess the possible side effects of VEGFC mRNA-LNP injection, we performed further experiments. First, we examined the possible off target effects in other organs. Second, we assessed blood vessel growth. Third, immune response was investigated.

For organ specificity studies, *Prox1*^{GFP} transgenic mice – where lymphatic endothelial cells express GFP – were injected with Poly(C) or VEGFC mRNA-LNP into the ears. The injected ears, contralateral ears, lungs and small intestines of Prox1^{GFP} animals were collected 22 days after injection, and GFP^{+} cells were quantified using flow cytometry. Significant increase in the number of GFP^{+} cells was found in the injected organ, but no difference was observed in other organs.

To examine the possible blood vessel proliferation, Poly(C) or VEGFC mRNA-LNP was injected into ears of mice. We found no significant difference in the number of blood vessels between Poly(C) RNA-LNP and VEGFC mRNA-LNP injected groups shown by CD31 (cluster of differentiation 31) and vWF (von willebrand factor) immunostaining 22 days after injection. CD31 is present on both lymphatics and blood vessels for it is a panendothelial marker. vWF can only be detectable on blood endothelial cells thus antibodies against it stain only blood vessels. In contrast, the increase of lymphatic vessel number was present as shown by LYVE1 and Podoplanin immunostaining.

To assess the possible immune cell infiltration, previously Poly(C) or VEGFC mRNA-LNP treated ears of *Prox1*^{GFP} mice were digested, then flow cytometry analysis was made against LYVE1 – to assess lymphatic endothelial cell number – or different immune cell specific markers such as: CD45 (cluster of differentiation 45) for all immune cells, Ly6G/C ((lymphocyte antigen 6 complex locus G6D/C) (GR1 (GPI-linked myeloid differentiation marker 1)) for neutrophil granulocytes, CD206 (cluster of differentiation 206) for macrophages and dendritic cells, CD3 (cluster of differentiation 3) for T cells, B220 (B cell marker 220) for B cells, and CD11b (cluster of differentiation 11b) for monocytes and many other immune cells. We made certain that we show only CD11b+ Ly6G/C- cells hence neutrophils are not gated again. There were significantly more lymphatic endothelial cells in the VEGFC mRNA-LNP treated ears compared to the controls, but there was no significant difference when the number of any assessed immune cells were compared.

Newly Grown Lymphatic Vessels Induced by VEGFC mRNA-LNP Are Fully Functional

We performed a number of additional experiments to examine the functionality of these newly formed lymphatic vessels.

70 kDa (kilodalton) Rh-D (Rhodamine-dextran) is a specific and efficient tool to examine lymphatic function. The interstitially injected macromolecules can be taken up and transported by lymphatics but not by the blood vessels. Thus, we injected fluorescently labeled macromolecules to make lymphatic function visible.

In our experiments Poly(C) RNA-LNP was injected to one and VEGFC mRNA-LNP to the contralateral ear of *Prox1*^{GFP} mice. 70 kDa Rhodamine-dextran was injected into both ears 22 days after the treatment. Fluorescent images were made by stereo microscopy. The green signal of Prox1^{GFP} and the magenta signal of the macromolecules were overlapping which indicated that the lymphatic vessels took up and transported the macromolecules.

Application of an *in vivo* Genetic Model to Induce Experimental Lymphedema in Mice

To examine the effect of VEGFC mRNA-LNP injection on lymphedema and demonstrate the proof-of-concept in a disease model we set up the *Flt4-CreER*^{T2}*; iDTR*$^{fl/fl}$ *in vivo* genetic mouse model in which Diphtheria Toxin injection results local deletion of lymphatic vessels and development of lymphedema.

Thickening and swelling of the paws and elevation of clinical score peaked 8 days after the first Diphtheria Toxin injection and this effect was present until up to 60 – in the case of clinical score – and to 75 days – in the case of width – post injection. To examine the histological changes specific to secondary lymphedema, we stained sections prepared from treated limbs with Hematoxylin and Eosin which showed the increase of fibroadipose area in Diphtheria Toxin treated paws. Fluorescent immunohistochemistry against lymphatic vessel specific markers such as, LYVE1 and Podoplanin showed the elimination of lymphatics 30 days after injection, and significantly lower number of lymphatics even after 75 days.

Functional analysis was performed by the injection of 70 kDa Rhodamine-dextran into the hind limbs of previously PBS (phosphate buffered saline) or Diphtheria Toxin treated animals. 30 and 75 days after injection mean fluorescent intensity of the popliteal lymph nodes showed decreased showing decreased uptake of the tracer molecule in the Diphtheria Toxin treated hind limb, while the signal was detectable in the popliteal region of the PBS treated animals. Quantification of the data showed significant reduction of the popliteal fluorescent signal in Diphtheria Toxin injected animals compared to PBS injected ones 30 and 75 days after the treatment.

Taken together, the presented results indicated that experimental secondary lymphedema was effectively developed in the *Flt4-CreER^{T2}; iDTR$^{fl/fl}$* mouse model.

VEGFC mRNA-LNP Reverses Lymphedema in an *in vivo* Genetic Mouse Model

After the development and setup of the genetic secondary lymphedema mouse model, 1 µg of Poly(C) or VEGFC mRNA-LNP was injected 8 days after the first Diphtheria Toxin injection of the paws. Paw thickness and clinical score of the lymphedema were significantly reduced 30, 60 and 75 days after the first Diphtheria Toxin injection.

Histological analysis showed that fibroadipose area, which is a sign of secondary lymphedema, was also reduced after VEGFC mRNA-LNP injection compared to control. Immunohistochemistry against LYVE1 and Podoplanin lymphatic vessel markers showed significant increase in the number of lymphatic vessels.

Functional analysis was performed by the injection of 70 kDa fluorescently labeled Rhodamine-dextran into the previously Poly(C) or VEGFC mRNA-LNP injected paws, that were previously treated with Diphtheria Toxin. Mean fluorescent intensity of the popliteal lymph nodes increased showing increased uptake of the tracer molecule in the VEGFC mRNA-LNP treated hind limb, while only minimal signal could be detected in the popliteal region of the Poly(C) mRNA-LNP treated animals. Quantification of the data showed significant growth of the popliteal fluorescent signal in VEGFC mRNA-LNP treated animals compared to Poly(C) RNA LNP injected ones.
In summary, VEGFC mRNA-LNP is an effective rescue therapy after Diphtheria Toxin injection in the genetic secondary lymphedema mouse model. Therefore, these findings demonstrate a proof of concept for VEGFC mRNA-LNP being an excellent tool to induce functional lymphatic vessel growth, and to reverse experimental lymphedema.

Dysfunction of lymphatics in the developing lung

Newborn *Clp1*$^{K/K}$ Mice Are Cyanotic, Show Signs of Respiratory Failure, and Die Shortly After Birth

In the next series of experiments, we set up and examined the *Clp1*$^{K/K}$ transgenic mouse model to assess organ-specific lymphatic function in the developing lung.
First, we set up timed mating between heterozygous adult animals. Embryos were removed from their mother by performing cesarean section at E19.5

(embryonic day 19.5). The genotype for the *Clp1* gene was verified with allele specific polymerase chain reaction (PCR).

Then, we examined newborn $Clp1^{K/K}$ mice and their viability and ability to breath. We found that $Clp1^{K/K}$ newborns were cyanotic and died shortly after birth, while control animals survived the intervention.

$Clp1^{K/K}$ Late Gestation Embryo Performs Less FBMs

Fetal breathing movements develop in late gestation mouse embryos and can be observed with ultrasound although this technique has great limitations.

We performed cesarean section in anesthetized mice, and removed the uterus from the abdomen. Then, we observed the movements of the embryos with ultrasound, and measured the number of fetal breathing movements during a 2-minute-long period.

We found that E18.5 $Clp1^{+/+}$ and $Clp1^{K/+}$ control embryos performed 2.67 ± 1.12 FBMs in 2 minutes (mean and SEM) (five out of six control embryos performed FBMs), while no FBM was found in one $Clp1^{K/K}$ embryo with normal heart activity. The diaphragm of the control animals slid, while the one of the $Clp1^{K/K}$ embryos did not move. There were 2 other litters at E18.5 which (control and non-control embryos overall) showed no FBM activity. Therefore, these animals were excluded from the study.

Collectively, our data indicate that $Clp1^{K/K}$ is a great genetic mouse model to study the role of FBMs and its physiologic effects on lymphatic vessels.

5. Conclusions

- We described a novel application of the nucleoside-modified mRNA-LNP therapeutic platform as an effective protein delivery system to trigger lymphangiogenic VEGFC expression.
- We showed that the administration of a single low dose of VEGFC mRNA-LNPs induces durable, organ-specific lymphatic growth, and formation of fully functional new lymphatic vessels.
- We designed and evaluated a novel gain of function approach for identifying the organ-specific physiological and pathophysiological roles of the lymphatic system.
- We showed that the nucleoside-modified VEGFC mRNA-LNP platform is effective in reversing disease progression in an experimental lymphedema mouse model by inducing the formation of a functional lymphatic network.

- We showed that *Clp1*$^{K/K}$ transgenic newborn mice are cyanotic, cannot breathe effectively, and die shortly after birth due to respiratory failure.
- We developed and applied an Ultrasound based technique to detect fetal breathing movements during the late gestation period in mice despite numerous technical challenges. Our results indicate that *Clp1* $^{K/K}$ embryos might perform less fetal breathing movements compared to controls.

6. Bibliography of the candidate's publications

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