IMPACT OF RENAL LYMPHATIC HYPERPLASIA ON BLOOD PRESSURE REGULATION IN MALE MICE

An Undergraduate Research Scholars Thesis

by

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Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Joseph Rutkowski

May 2017

Major: Chemistry, B.S.

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ABSTRACT

Impact of Renal Lymphatic Hyperplasia on Blood Pressure Regulation

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Chronic high blood pressure, or hypertension, is identified as a risk factor for heart disease, stroke, and chronic kidney disease. The primary cause of most hypertension is increased peripheral vascular resistance that is controlled, in large part, by the kidney's water handling. In the kidney, specific immune cell subsets and overall renal inflammation have both been identified as drivers of hypertension in preclinical models. Lymphatic vessels serve as a route of both fluid and immune cell clearance and their expansion, lymphangiogenesis, is necessary for the resolution of tissue inflammation. We hypothesized that by increasing renal lymphangiogenesis, renal inflammation would be reduced and blood pressure would be normalized during salt-sensitive hypertension. To investigate the role of renal immune cell trafficking in instances of blood pressure challenge, we employed a murine model of inducible renal lymphatic expansion. Mice expressing a lymphatic growth factor, vascular endothelial growth factor (VEGF)-D, under the control of a TRE-promoter were crossed with mice expressing a Kidney Specific Protein-regulated transactivator (rtTA). Upon administration of doxycycline, the rtTA-dox complex binds to the TRE promoter region, causing transcription of VEGF-D only in renal tubular epithelial cells. The resultant kidney-specific VEGF-D

overexpression caused expansion of the existing renal lymphatic network in addition to generating de novo lymphangiogenesis in the cortex of “Kid-VD” mice. We then utilized the Kid-VD mouse model during an established rodent hypertension regimen of nitric oxide inhibition and high salt diet loading to identify the impact – through weekly blood pressure measures - and mechanism – by cellular, protein, and RNA analysis – of expanded renal lymphatics on blood pressure regulation. Lymphatic circulation may thus provide a new target for the treatment of chronic hypertension and its associated co-morbidities.

DEDICATION

I would like to dedicate this research study to all of the individuals who have invested in me as a student, as an individual, and as a scientist. Thank you for your dedication, patience, and for sharing your love of science with me.

ACKNOWLEDGEMENTS

Throughout my time at Texas A&M I have received help and support from many colleagues and superiors. Firstly, I would like to thank Dr. Joseph Rutkowski for his enduring patience, wise guidance, and continual teaching throughout the course of this research.

I would also like to thank Dr. Mitchell and Catalina Lopez for their assistance and collaboration in this study. Additionally, Sheridan and Andi were responsible for maintaining the mice used in this study, making this study possible.

Along every step of the way, however, my friends and family have supported me and I am ever-grateful for their kindness.

ABBREVIATIONS

VEGFR-3	vascular endothelial growth factor receptor 3
LYVE1	lymphatic vessel hyaluronan receptor 1
LEC	lymphatic endothelial cell
IAL	inflammation associated lymphangiogenesis
dox	doxycycline
L-NAME	L-nitro-arginine methyl ester
KSP	kidney-specific protein
rtTA	reverse tetracycline transactivator
qPCR	quantitative polymerase chain reaction
VEGF	vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Lymphatics History

Compared to many other areas of study, lymphatics are understudied, underfunded, and undervalued. Despite documentation of what is understood to be the lymphatics in the 4th century B.C., it was not until the mid-16th and 17th centuries A.D. that significant strides in the field were made[1]. During this time period an increase in the accepted practice of human cadaver dissections allowed for significant anatomical descriptions of this system, though physiological conclusions were merely associations with ascites, edema, and cancer metastasis.[1,2] More surgical approaches to studying lymphatics nearly 100 years later led to the conclusion that the system is primarily responsible for absorption,[1] specifically of fluid extravastated from their cardiovascular counterparts. This process of fluid leakage was eventually determined by Ernest Starling who described the relationship between hydrostatic and oncotic pressure that drives the fluid to leave the blood vasculature in the first place.[3] Now, there is a general understanding that the lymphatic system is responsible for more beyond returning lost fluid from the interstitium and plays key roles in antigen recognition and response, macromolecular transport, and several pathological states beyond cancer metastasis. Despite these important functions, research in the field of lymphatics has been hindered by limited lymphatic endothelium-specific markers. Relatively recently, markers such as vascular endothelial growth factor receptor-3 (VEGFR-3), podoplanin, lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1), and pospero homeobox 1 (prox1) were identified to be reliable lymphatic endotheial cell (LEC) identifiers.[4] Despite these

challenges to discovery, however, the increasingly prominent function of this system in numerous diseases necessitates increased investigation and is the fundamental basis of this study.

Basic Functions of the Lymphatic System

The lymphatic system plays several key roles in the body that are necessary for maintaining homeostasis. The first of these functions is that of fluid regulation. The lymphatics are fundamentally responsible for returning fluid leaked by the blood vessels into system circulation. This begins with the loss of fluid from the blood vasculature due to differences in hydrostatic and oncotic pressures between the blood and interstitium.[1] As this interstitial fluid is taken up by the lymphatic vessels, it is transported along with certain cells and macromolecules through the lymphatic network (and several lymph nodes) until it passes into the thoracic duct is returned to system circulation through the inferior vena cava.[5] A majority of the cells taken up in the lymph are macrophages and dendritic cells which present antigens to the draining lymph node, stimulating an immune response when necessary.[6-8] Additionally, the lymphatic vessels are responsible for taking up fatty acids in the gut and transporting for processing and later use.[9] These represent the homeostatic roles of the lymphatic system.

Lymphatics and Inflammation

The importance of the lymphatics extends far beyond homeostatic regulation, however, and enters pathological conditions where the lymphatic system can be praised for assisting in the resolution of the disease state, or blamed for worsening it. Whether the result is beneficent or harmful depends on the balance between different functions of the system. Primarily, as previously stated, the major function of the network is that of fluid collection, therefore it is

reasonable to conclude that in instances of edema, ascites, and general conditions of fluid retention, the lymphatics present an escape for fluid buildup.[1] Simultaneously, however, lymph nodes are responsible for producing responses to the immune cells received from the periphery, complicating the seemingly beneficial lymphatic vessels.[10] Consider the case of inflammation (of primary interest in this study). Within the cocktail of cells and chemicals taken up by the lymphatics are both pro- and anti-inflammatory cytokines including interleukin1 (IL-1), IL-10, respectively.[11] This requires the resulting bodily response to be a balance between the seemingly contradicting cells presented to the lymph nodes. Moreover, whether the response supports or hinders inflammation is further compounded by the amount of cytokine, its target cell, and the timing.[11] Furthermore, the issue of the role of the lymphatic system in inflammation is compounded by the presence of a process appropriately termed inflammation-associated lymphangiogenesis (IAL). IAL is the process by which the existing lymphatic vasculature is expanded or a new vasculature is created *de novo* in response to pro-lymphangiogenic factors secreted by immune cell subsets present in conditions of inflammation.[12] Along with these factors such as vascular endothelial growth factor C (VEGF-C) and VEGF-D, are factors that are anti-lymphangiogenic, such as interferon gamma (IFNγ) produced by T-cell subsets.[13,14] The balance between these two groups is believed to determine the extent of IAL observed, the degree of benefit/harm, and the newly-formed network's eventual regression (if any).[12,13,15,16] Setting and cause also play a significant role in IAL and its resulting effects. For example, IAL is known to both occur and be harmful to keteroplasties,[17-20] cardiac transplants[21] and other allografts, beneficial under conditions of both chronic and acute skin inflammation[22,23]and myocardial infarctions,[24,25] and ambiguous in the realm of airway inflammation[26] and renal inflammatory pathologies.[27] This necessitates the question of whether IAL can be targeted as a

therapeutic in certain pathological instances, which is what several groups are currently investigating. A summary of the effects of IAL in various tissues can be found in this review[28] (**Appendix**), and is graphically represented in **Figure 1.**

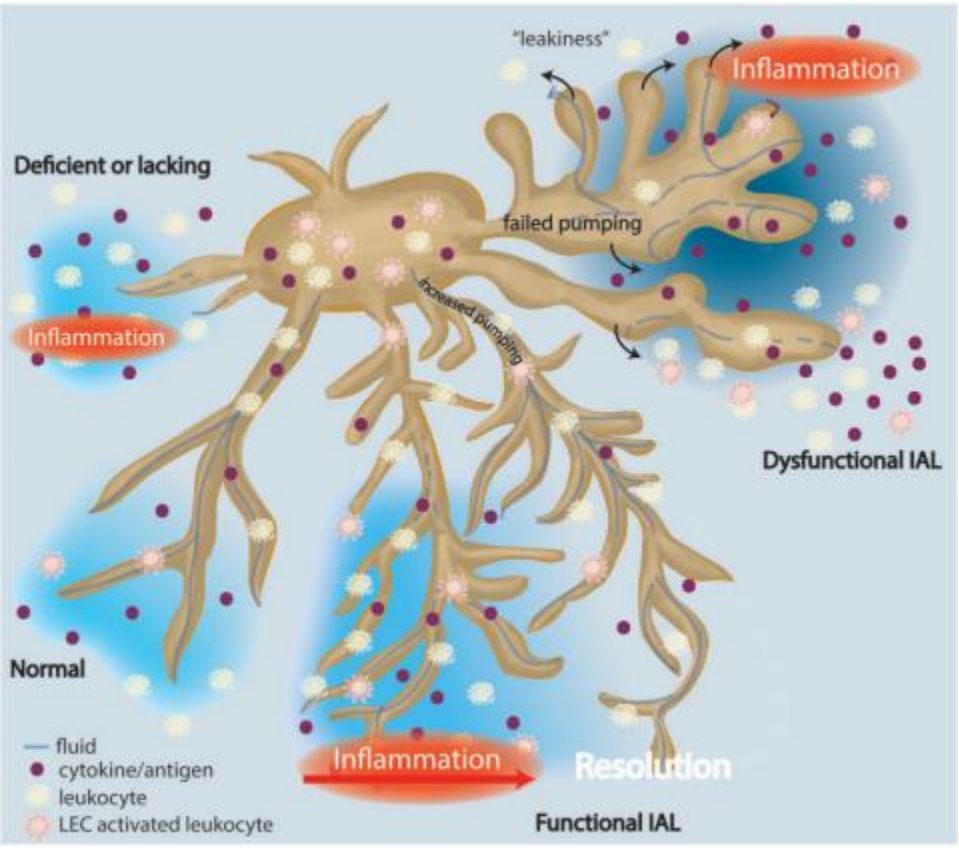

Figure 1. A schematic depicting the potential effects of IAL. Instances of inflammation can induce lymphangiogenesis with particular chemokines. The new lymphatics then serve as an exit route for fluid, immune cells, and cytokines resulting in resolution of the inflammation. In instances of inflammation when the lymphatic vasculature does not expand, however, this can lead to unresolved inflammation. In times when the lymphatics expand and the resulting network is dysfunctional, this can worsen the pathology and result in persistent inflammation. Figure reprinted with permission from Abouelkheir *et al.* **2017.** 884-895.[28]

Inflammation and Hypertension

One particular pathological state becoming increasingly frequent as a secondary complication of diseases such as diabetes and affecting a large portion of the population and having a negative compounding effect on health is hypertension.[29] Hypertension is a disease characterized by persistently increased blood pressure in which inflammation and immune cells have implicated as key players.[30] Examples of these influential participants include Th17 cells which are pro-inflammatory and pro-hypertensive, in contrast to regulator T-cells (Tregs) which play the opposing roles, implicating an immune basis to the intimate relationship between inflammation and hypertension.[31] Recently, De Miguel *et al.* investigated the infiltration of immune cells (particularly T lymphocyte subsets) into the kidney in cases of hypertension (specifically Dahl salt-sensitive hypertension).[32] Considering the role of the lymphatic system in immune cell trafficking and inflammation, in addition to the role of immune cells and inflammation in the development of hypertension, this begs the question of whether IAL is at play in cases of hypertension. To investigate this, our group sought to generate an expanded renal lymphatic network in murine subjects[33] submitted to various blood pressure challenges to determine any observable effect.

CHAPTER II

METHODS

Experimental Design

Pathway of Importance

To choose a useful mouse model, it is important to note the signaling pathway of interest. In this case, the desired result is an expansion of the lymphatic network, a process achieved through VEGFR-3 signaling.[33] The primary ligands for VEGFR-3 are VEGF-C and VEGF-D-secreted growth factors.[34] It is important to note that while both of these ligands also participate in VEGFR-2 signaling on blood endothelium in humans, murine VEGF-D specifically binds only VEGFR-3 in the mouse.[34] Therefore, to exclusively stimulate lymphangiogenesis in the murine models, it is important to do so through VEGF-D signaling only, which was the model used in this study, generated and maintained by the Rutkowski group at the Texas A&M Health Science Center.

The Mouse Model

To perform the mouse model, mice having VEGF-D under the control of a tetracycline response element (TRE) promoter were crossed with mice having a tissue specific reverse tetracycline transactivator (rtTA) by the Rutkowski group.[33] The resulting offspring, once induced with doxycycline, will then have overexpression of the protein VEGF-D, stimulating lymphangiogenesis in the tissue of interest (**Figure 2**). In this study, the rtTA is under the control of Cdh16, or the kidney specific protein (KSP), causing the rtTA to be expressed throughout the

renal tubular epithelium.[35] The kidney was chosen due to recent evidences of pro-inflammatory immune cell infiltration of the kidney in response to hypertension.[32]

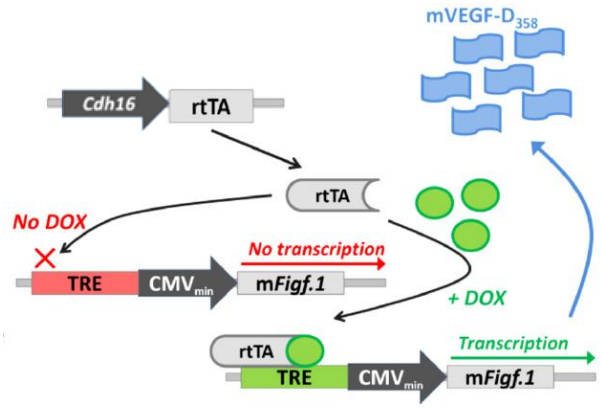

Figure 2. **Scheme depicting genetic construction of VEGF-D overexpressing mouse.** In this study, doxycycline was added to the drinking water given to the mice. (*Adapted with permission from Lammoglia* et al. **2016.** H384-H394.[33]*)*

The Experimental Conditions

Mice carrying the TRE-VEGF-D transgene were crossed to mice having a kidney-specific protein rtTA (KSP-rtTA). Mice from the resulting litter were genotyped (confirmed by qPCR analysis) for the presence of the rtTA and VEGF-D transgenes. Mice containing both transgenes, "Kid-VD" mice, were the test subjects with "WT" controls lacking one of the transgenes (and, hence, no inducibility). All experiments were performed with adult male mice only. For the extent of the study, all ten male mice from the three litters received water containing 200 mg/mL doxycyline. This water was remade twice weekly. After one week, five of the mice received water containing both 200 mg/mL doxycycline and 0.5 mg/mL L-Nitro-Arginine Methyl Ester (L-NAME), a nitric oxide (NO) inhibitor that increases blood pressure. The other five mice continued receiving water containing only doxycycline. This procedure

resulting in four total conditions: Kid-VD mice receiving dox water only (n=3), WT mice receiving dox water only (n=3), Kid-VD mice receiving dox and L-NAME water (n=3) and WT mice receiving dox and L-NAME water (n=2). At the end of four weeks, the mice were sacrificed and their kidneys were collected for further analysis. All animal study protocols were previously approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Another cohort of KSP-rtTA mice was bred for a second blood pressure measurement experiment. This cohort consisted of 8 Kid-VD positive male mice, 5 control male mice, 3 Kid-VD positive female mice, and 3 control female mice (one Kid-VD female mouse was lost on week 6 of the experiment). All these mice received 0.5 mg/mL L-NAME water for 2 weeks, followed by one week of tap water, and 200 mg/L dox water for 4 weeks. These mice received normal chow for the first 4 weeks, and high-salt chow (4% NaCl) after one week of dox for the final 3 weeks. See **Table 1** for a summary of this protocol. After this time point all of the mice were sacrificed, and their kidneys and renal lymph nodes were collected for further analysis. This protocol is expected to result in hypertension for blood pressure measurements of weeks 1-2, a slight elevation in blood pressure from baseline weeks 3-4, and hypertension (higher than weeks 1-2) for weeks 5-7. All animal study protocols were previously approved by the Institutional Animal Care and Use Committee at Texas A&M University.

Table 1. Table depicting water and feed conditions for second blood pressure experiment.

	Water	Feed
Week 0	L-Name	Normal
Week 1	L-Name	Normal
Week 2	Tap	Normal
Week 3	Dox 200	Normal
Week 4	Dox 200	High Salt
Week 5	Dox 200	High Salt
Week 6	Dox 200	High Salt
Week 7	Sac	Sac

Immunofluorescence

Immediately following tissue harvesting, one kidney was fixed in formalin and sent to the Texas A&M VIBS Histology Laboratory where the samples were embedded in paraffin and sectioned sagitally in 3 μm-thick sections. Sections were deparaffinized using xylenes and rehydrated using a series of ethanol solutions. Subsequently, tissues were labeled with primary antibodies to murine lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) (goat polyclonal; R&D Systems) and podoplanin (goat polyclonal; R&D Systems) on separate sections. Secondary labeling was achieved with Alexafluor 488-labeled donkey antibodies (Life Technologies, Carlsbad, CA). Immunofluorescence images were then obtained using a Nikon Eclipse E600 microscope. Images were captured using NIS-Elements imaging software.

RNA Preparation and Quantitative Real-Time RT-PCR

Immediately after harvesting, one quarter of each kidney (containing both cortex and medulla) was flash frozen in liquid N_2 for RNA analysis. For RNA extraction, samples were ground into a fine powder using a mortar and pestle and the extraction was performed using a

Qiagen RNeasy Mini Kit, following the contained manufacturer's instructions (Qiagen, Germantown, MD). cDNA was obtained from 1 ug of the extracted RNA using the iScript cDNA Synthesis kit instructions (Bio-Rad Laboratories, Hercules, CA). The resulting cDNA was mixed with Power SYBER Green PCR Master Mix (Applied Biosystems) with the corresponding gene primers on an Applied Biosystems 7900 quantitative PCR machine for quantitative real time RT-PCR relative expression analysis (see **Table 2** for primer sequences). Values were normalized to ubiquitin expression and subsequently to the corresponding expression of the WT mice of the same experimental condition. Statistical significance was determined using a Student's T-test with a level of significance set to 0.05.

Table 2a. Table containing forward sequences for primers used in qPCR analysis. Primers not listed were obtained from a collaborating lab.

Gene	Forward
CD11c	GATGCGGTGGAACACTTTCT
CD3e	ACCAAGAAGAGCGACTTCCA
CD4	CTTTGGCTATGGGCTTCCAGTC
CD8a	CGGCGGCCTGTTTGCAGT
F480	ACAAAGAAGTGGCCGAGAGA
FGFR1	GAGAAAGTCAACCTCCTCTCTG
FoxP3	GCTCTTACTGACTGGCATGAG
GATA3	CCGTTGACCCGCTTTCTGT
IL10	CTGGATAGCCTTTCTTCTGCTG
IL6	TCCTAGCTGTCACTCAAGGGA
LYVE1	CTACCGGGTTCGGATGTAAGTC
mKlotho	GGTCCACAGACATCATGGAA
TNFa	GCCCTGCTGTGGTCTCACTAC
Ubiq	ATCAGAAGATCGGGCGCTGTTGTA
VEGFA	GGAGATCCTTCGAGGAGCACTT
VEGFC	CTGACAAGCAGTTTCAGGCTTGGT
VEGFD	AAATCGCGCACTCTGAGGA
VEGFR2	GAGGATACCACTCCCAACAGACC
VEGFR3	GCCCAGTGTTACCACCAAGAAG

Table 2b. Table containing reverse sequences for primers used in qPCR analysis. Primers not listed were obtained from a collaborating lab.

Gene	Reverse
CD11c	ACTGTCCTCGACTTCCGAGA
CD3e	AACCAGGAGAACCCCAGAGT
CD4	GCAAGGAGGACAGAGTTTATCGTG
CD8a	TTGTGGCGGATGGCATTCTTC
F480	CGGTGAAATAGGGCAAAAGA
FGFR1	GAAGACTCCTCCCAGGTATATG
FoxP3	CGCAGCTCTAGGAGCATGTG
GATA3	CGGCGTCCATTTTCTTTGGAA
IL10	GCACACTGTGTCCGAACTC
IL6	TCAGAGAACTTCCAGGTGAAGA
LYVE1	GTTCACACACTCCCTGCCTCT
mKlotho	CAGTGTCAGGCAGCTAACAAG
TNFa	CAAAGCATTGCCCATTCGAT
Ubiq	TGTGTCATGTCCGCCCTTCAGTTA
VEGFA	GGCGATTTAGCAGCAGATATAAGAA
VEGFC	TTCAGCCCACACTCCGCTATACAT
VEGFD	TGGCAAGACTTTTGAGCTTCAA
VEGFR2	AAGTGCATCATCGTTGTTCATA
VEGFR3	GCTCTTTTTAGATACTGTGGTGAGGAA

CHAPTER III

RESULTS

L-NAME Study

qPCR and Immunofluorescence Confirm Overexpression Model

Quantitative RT-PCR data was obtained for the relative gene expression of VEGF-D for confirmation of overexpression for the Kid-VD mice receiving doxycycline. When normalized to ubiquitin and wild type murine expression within each experimental group (receiving dox only water or water containing both dox and L-NAME), the relative expression for both conditions is significantly elevated for the Kid-VD mice (**Figure 3**). This data confirms the VEGF-D overexpression model established in these transgenic mice.

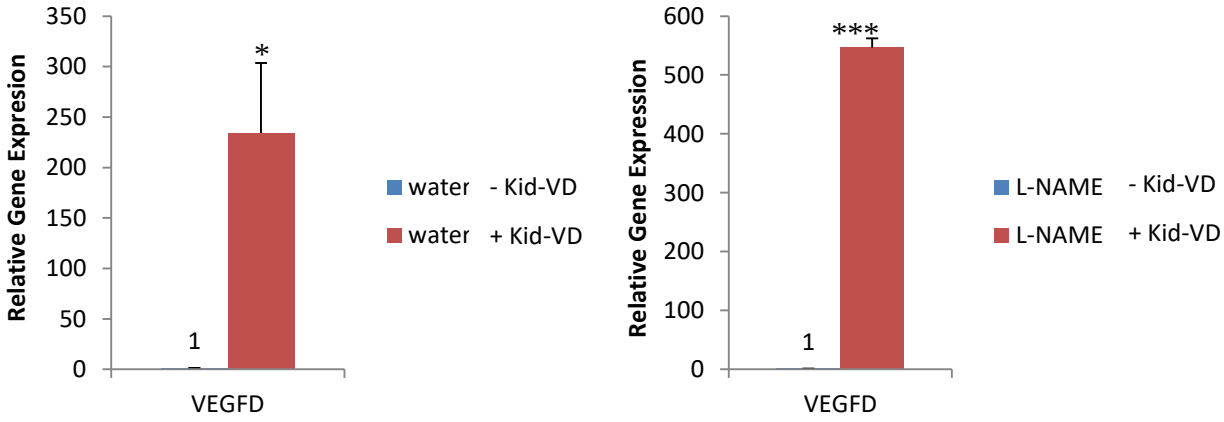

Figure 3. Quantitative real-time RT-PCR confirms VEGF-D overexpression model. Relative VEGF-D expression of mice positive for KSP-rtTA (red) relative to WT mice (blue) with same water treatment. Significant overexpression of VEGF-D in both experimental conditions confirms validity of dox-dependent overexpression model used in this study. Mice that did not receive L-NAME received 200 mg/mL dox water for four weeks and mice receiving

L-NAME were given 200 mg/mL dox only water for one week followed by water having 200 mg/mL dox and 0.5 mg/mL L-NAME. It should be noted that baseline VEGF-D levels for the L-NAME cohort were approximately half that of the dox only cohort. Asterisk corresponds to a value of statistical significance (*$p<0.05$,***$p<0.001$ using a two-tailed students' T-test with unequal variance).

As previously described, murine VEGF-D signaling occurs only through the VEGFR-3 pathway, which should result in an increase in lymphatic vasculature only. To confirm that the increase in signaling expanding the lymphatic vessels rather than simply indicated an increase in immune-cell signaling, qPCR analysis was performed for the lymphatic markers LYVE-1 and VEGFR-3 (**Figure 4**). Podoplanin, while often a lymphatic marker, was not used in the qPCR analysis because of its constitutive expression by podocytes in the kidney. The increase in both LYVE-1 and VEGFR-3 expression supports that the VEGFR-3 signaling that is occurring is primarily through the lymphatic vasculature rather than simply the immune cell population present, as is expected from the renal lymphatic hyperplasia model. Additionally, the overexpressed lymphatic markers indicate that the increase in VEGF-D is not solely due to its role as a chemokine.

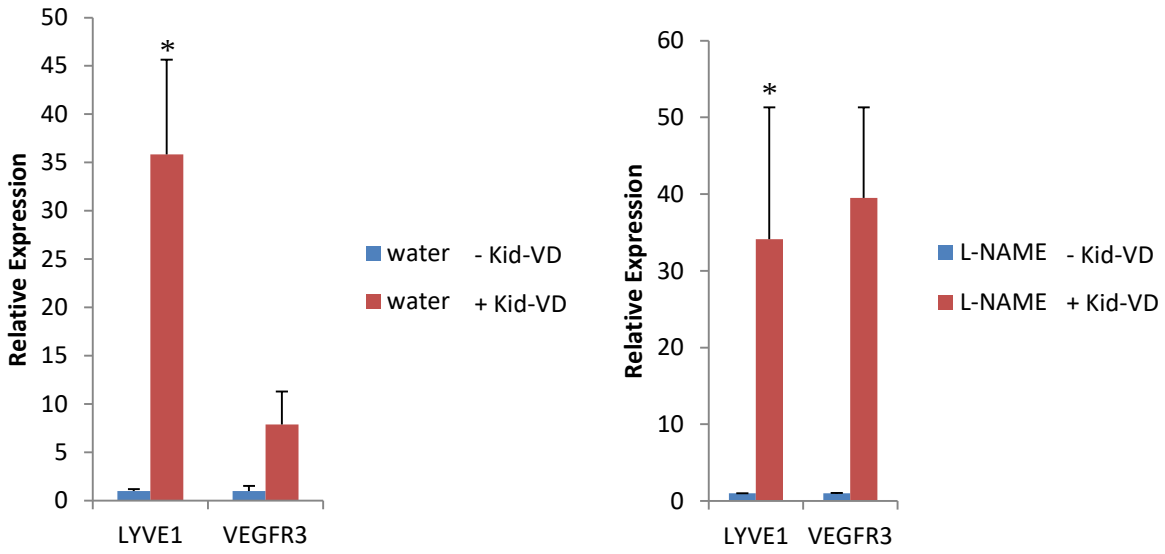

Figure 4. Quantitative real-time RT-PCR confirms lymphatic vessel expansion. Relative LYVE-1 and VEGFR-3 expression for mice receiving dox water only (left) and mice receiving water supplemented with dox and L-NAME (right). The increase in both of these lymphatic markers supports the renal lymphatic hyperplasia model and confirms lymphatic expansion rather than solely immune cell signaling. Asterisk corresponds to a value of statistical significance (*$p<0.05$ using a two-tailed students' T-test with unequal variance).

Although there are previously published reports that there is no direct change in blood endothelium with the VEGFR-3 signaling in the adult.[33] there have been studies demonstrating that renal blood endothelium is potentially VEGFR-3 positive.[36] Therefore, it is important to rule out blood vessel expansion as a factor in our study. To do this, we looked at the relative expression of VEGFR-2, VEGF-C, and CD-31 (see **Figure 5**). VEGFR-2 is a VEGF receptor found primarily on blood endothelium that can be signaled by VEGF-C (although VEGF-C is primarily lymphangiogenic and signals via VEGFR-3).[33] The lack of significant change in VEGF-C levels confirms the model of inducing lymphangiogenesis is through overexpressing

VEGF-D specifically.[33] VEGFR-2 and CD-31 levels were used to mark any observable blood angiogenesis. Their elevated expression requires further investigation, however considering that these markers are also expressed to a lesser extent in LECs.[37] the massive increase in LEC numbers observed in the model may be sufficient to explain the overexpression.

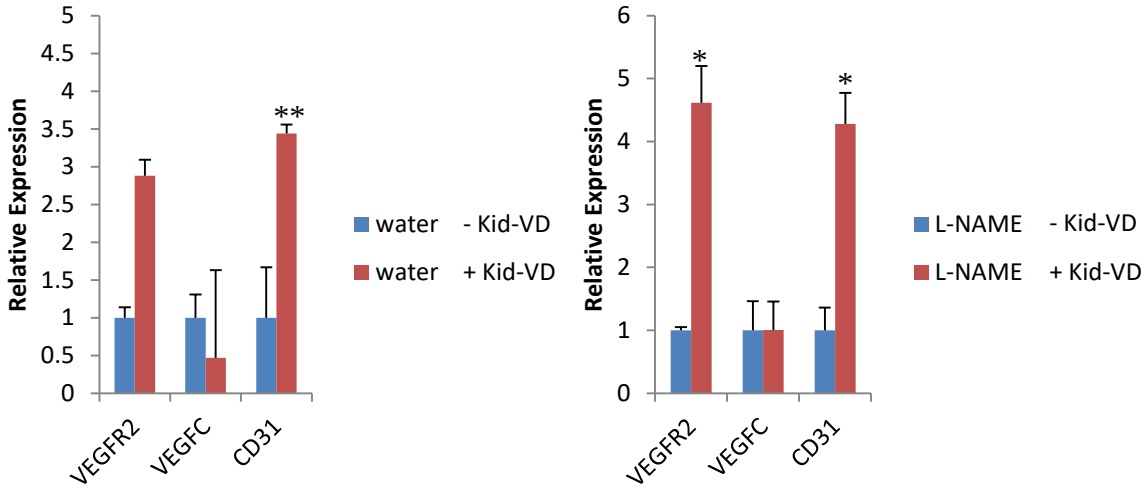

Figure 5. Slight elevations in endothelial markers can be explained by extreme lymphatic overexpression. qPCR expression analysis for primarily endothelial cell markers. Despite some change in expression levels, these are less than the approximately 50-fold expression observed for lymphatic markers, the expression in these markers can be attributed to the increased presence of lymphatics considering that all three of these markers are concurrently expressed on lymphatic endothelial cells (though in small quantities). Asterisk corresponds to a value of statistical significance (*$p<0.05$, **$p<0.01$ using a two-tailed students' T-test with unequal variance).

To further confirm the presence of lymphatic expansion, immunofluorescence was performed to observe the morphology of the newly formed lymphatics (see **Figure 6**). As is

easily observed from the images, significant lymphatic expansion occurs for Kid-VD mice compared to WT. Future studies need to be conducted to both quantify this expansion and determine the functionality of these newly formed vessels. Based on the very limited presence of cortical lymphatics in control mice, however, it can be assumed that the lymphatic expansion observed is the generation of a new lymphatic network rather than merely dilation or hypertrophy of previously present vessels.

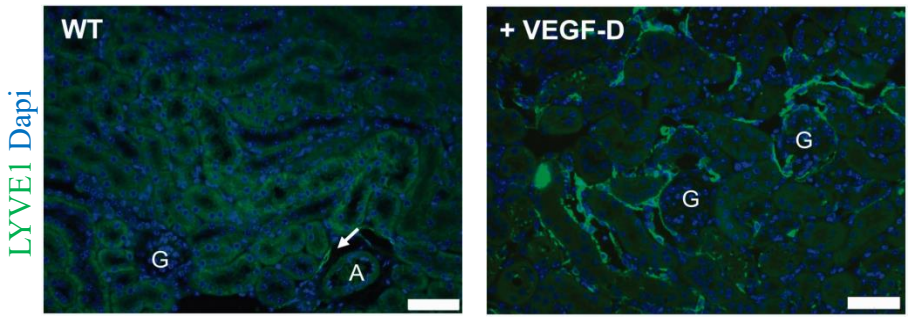

Figure 6. Immunofluorescence demonstrates lymphatic expansion. Images above were taken from the renal cortex of WT mice (left) and Kid-VD mice (right). Images were taken at 40X magnification, scale bars being 50 µm. (LYVE-1 green, dapi blue). These images morphologically demonstrate an expansion of the lymphatic network. (The arrow corresponds to a lymphatic vessel, A-arteriole, G-glomerulus). Images reprinted with permission from Abouelkheir *et al.* **2017.** 884- 895.[28]

Preliminary Blood Pressure Data Suggest a Protective Effect of Lymphatic Expansion on L-NAME Induced Hypertension

To observe the effect of lymphatic expansion on blood pressure, an intersection previously published by Mattson *et al.*, systolic blood pressures were measured on a weekly

basis for each experimental condition (**Figure 7**)[38]. Consistently for both groups, Kid-VD mice showed a decreased blood pressure from week 2-after those receiving L-NAME had been on the treatment for one week. Admittedly, none of the blood pressure values are statistically different. Therefore, this study needs to be repeated with a greater sample size to determine if the effect is reproducible. Furthermore, negative control mice showed slightly elevated blood pressure compared to what is to be expected for a mouse (90-110 mmHg). This could indicate that the data is skewed and supports the need for repeated experiments. Additionally, a time course study needs to be performed to determine how long it takes for the lymphatic vasculature to expand.

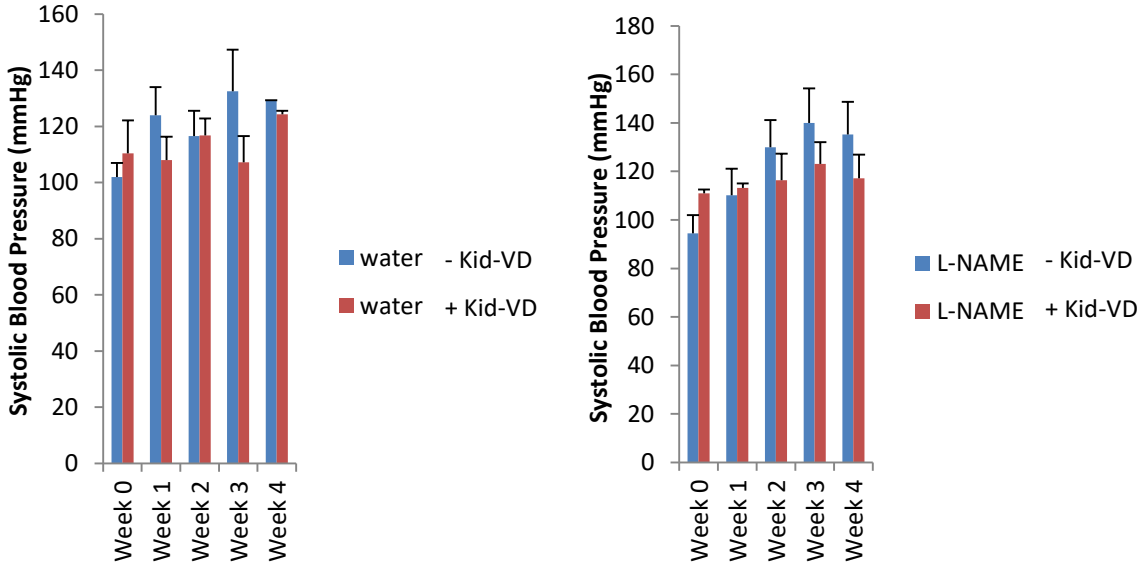

Figure 7. Systolic blood pressure measurements suggest a slightly protective effect. Systolic blood pressure measurements for WT and Kid-VD mice receiving dox only water or dox water followed by dox and L-NAME water (given from week 2). Week 0 measurements were obtained immediately prior to starting the study. From week 2 onward, both Kid-VD show a slightly protective effect and lowered blood pressure.

qPCR Data Show a Slightly Protective Effect in Mice Not Receiving L-NAME in L-NAME Induced Hypertension Study

qPCR analysis was performed for several immune cell populations and inflammation markers for both conditions (**Figure 8**). In the dox-only Kid-VD mice, IL1B, a pro-inflammatory marker was significantly decreased, supporting the hypothesis of a protective role of lymphatic expansion in instances lacking significant challenge. Moreover, klotho, a protein associated with kidney welfare, was significantly elevated in these mice.[39] Other seemingly elevated or decreased values were not statistically significant, however. This could be due to the small sample sizes of each group and require the experiment to be repeated to establish reproducibility and determine significance.

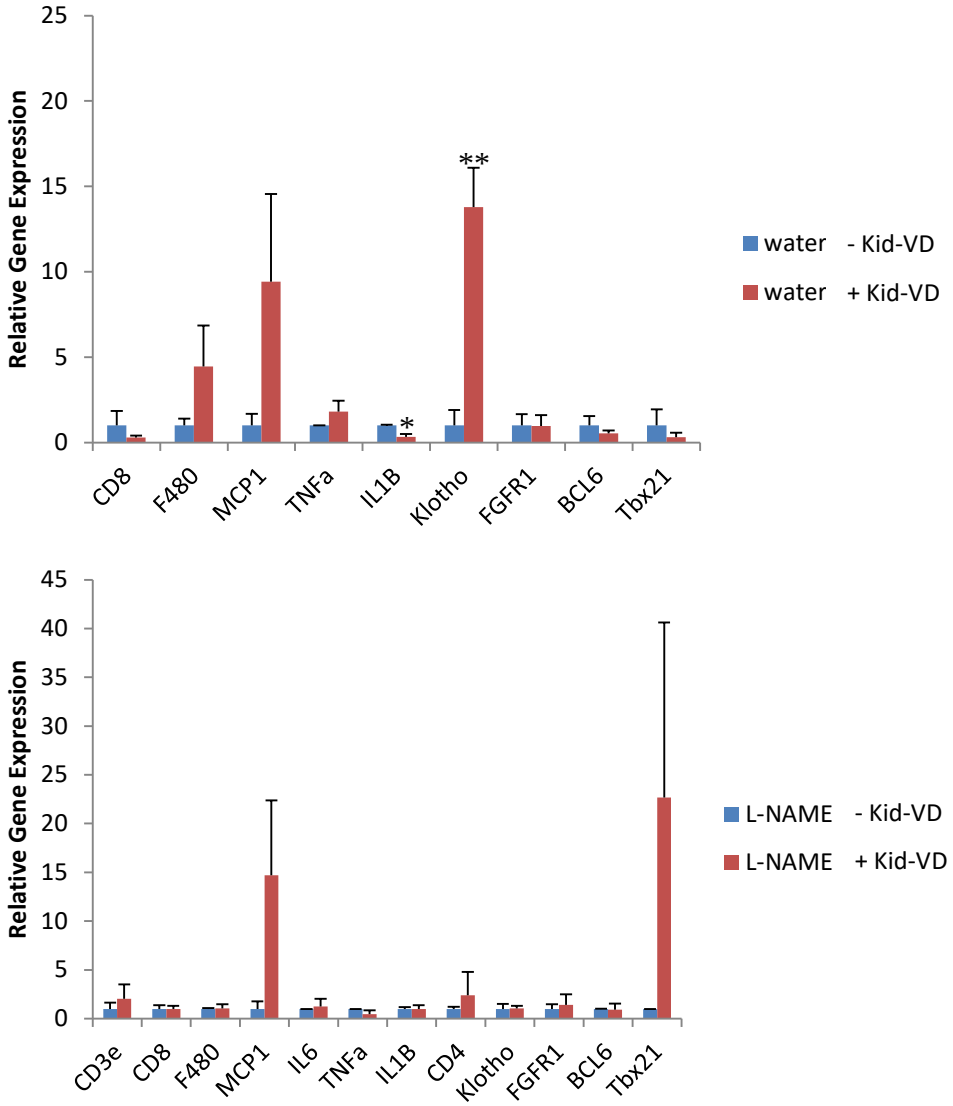

Figure 8. Relative gene expression analysis shows a decrease in pro-inflammatory marker IL1B and an increase in protection-associated protein, klotho, in Kid-VD mice. Relative gene expression data obtained by qPCR for control and experimental mice receiving dox only water (top) and water supplemented with both dox and L-NAME. Relative expression, normalized to ubiquitin, shows a decrease in IL1B and an increase in klotho in mice receiving only dox water. No significant differences were seen in the mice receiving L-NAME treatment.

No statistical test could be performed for IL6 and TNFa. Otherwise, a two-tailed students' T-test with unequal variance was performed to determine statistical significance (*p<0.05, **p<0.01).

Salt-Sensitive Hypertension Study

qPCR and Immunofluorescence Confirm Overexpression Model

Subsequent to sacrificing the cohort of mice suffering from salt-sensitive hypertension, qPCR analysis was confirm overexpression model and the presence of lymphatic vessels. After normalizing to ubiquitin and the expression of WT mice, results show statistically significant overexpression of VEGF-D (**Figure 9**) and its receptor, VEGFR-3, along with increased expression of lymphatic marker, LYVE-1 (**Figure 10**). The increased expression of LYVE-1 along with the receptor and its ligand confirm the lymphatic hyperplasia model rather than merely increased immune signaling.

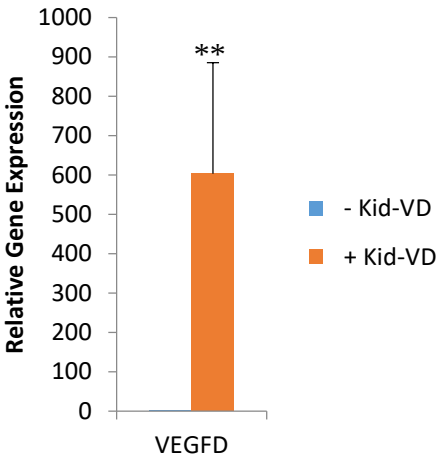

Figure 9. qPCR analysis of VEGF-D expression confirms overexpression model. Relative VEGF-D expression of Kid-VD mice (red) relative to WT mice (blue) with same protocol. Significant overexpression of VEGF-D in experimental condition confirms validity of dox-

dependent overexpression model used in this study. (**p<0.01, using a two-tailed students' T-test with unequal variance).

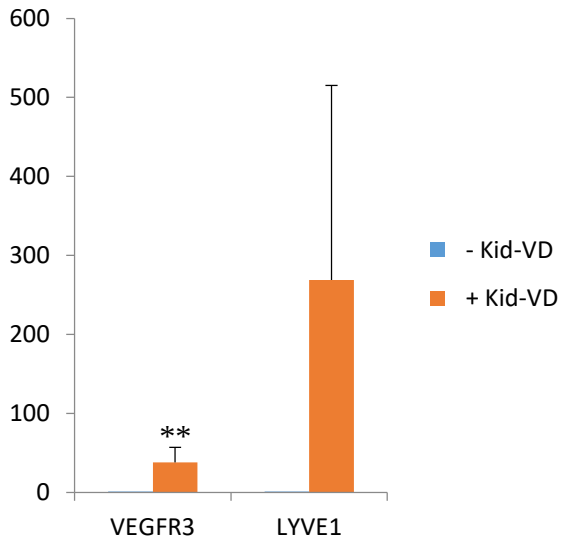

Figure 10. qPCR analysis of lymphatic markers confirm overexpression model. Significant overexpression of VEGFR-3, the VEGF-D receptor, combined with increased expression of lymphatic marker LYVE-1 confirm the lymphatic hyperplasia model. (**p<0.01, using a two-tailed students' T-test with unequal variance).

Once lymphatic hyperplasia was confirmed, the next task was to determine if there was any effect on blood angiogenesis. To this effect, qPCR analysis was performed for VEGF-C, VEGFR-2, CD31, and VEGF-A. VEGF-C is a VEGF isoform expressed by both blood endothelium and lymphatics that signals through VEGFR-2. CD31 is an endothelial marker, and VEGF-A is a blood-endothelium specific isoform of VEGF-D. As the relative expression of these genes illustrates, (**Figure 11**) there are only significant increases in VEGR-2 and CD31. Considering that VEGFR-2 is also expressed by lymphatic vessels, the VEGFR-2 overexpression

can be explained by the expanded lymphatic vasculature. The source of the increased CD31 expression levels, however, needs to be determined by further experimentation. The expression levels of VEGF-C and VEGF-A, however, are comparable to the mice in the study lacking VEGF-D overexpression, supporting the utility of the model in its specificity for VEGF-D and the conclusion that the increased CD31 levels are likely not due to an increase in overall angiogenic program.

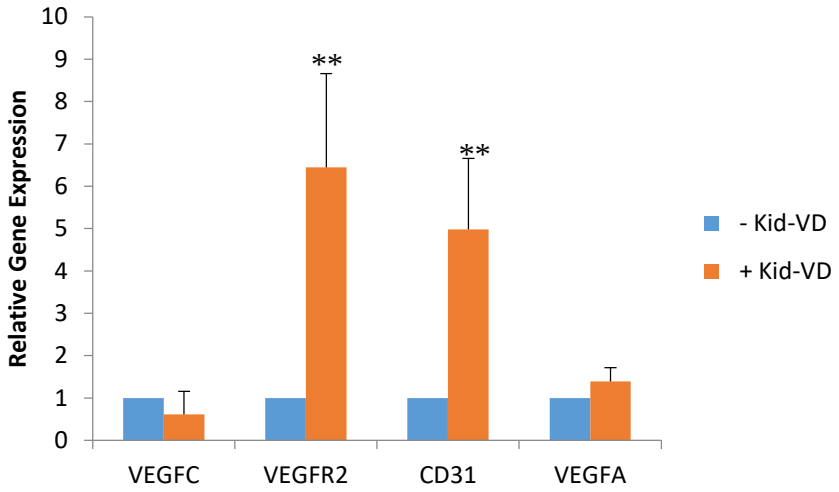

Figure 11. qPCR analysis of blood vasculature markers is likely not due to expanded blood vasculature. qPCR analysis of VEGF-C, VEGFR-2, CD31, and VEGF-A shows significant elevation of VEGFR-2 and CD31. Increased VEGFR-2 levels can be explained by the expanded lymphatic vasculature while the source of increased CD31 needs to be determined through further studies. (**$p<0.01$**,** using a two-tailed students' T-test with unequal variance).

To morphologically confirm the presence of an expanded lymphatic network, immunofluorescence was conducted to mark lymphatic vessels using LYVE-1 (**Figure 12**). As the images depict, mice having the overexpression model have many more LYVE-1 positive

structures compared to mice lacking this genetic modification. Expanded lymphatic vasculature was most prominent in the cortex. While this serves to confirm the overexpression model, more studies need to be conducted to quantify the expansion and determine the functionality of the expanded network. These are studies we hope to conduct in the future. The degree of expansion, however, is suggestive of *de novo* lymphangiogenesis rather than a mere expansion of the preexisting network.

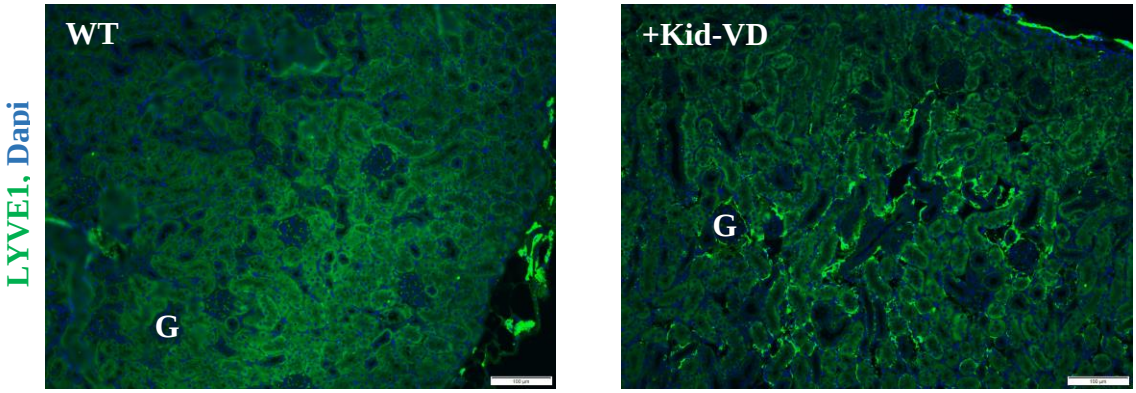

Figure 12. Immunofluorescent histology of + Kid-VD mice shows expanded lymphatic network, confirming hyperplasia model. Images were taken from the renal cortex of WT (left) and Kid-VD (right) mice. Images were taken at 20X magnification, scale bars being 100 μm. (LYVE-1 green, dapi blue, G-glomerulus).

Blood Pressure Data Show + Kid-VD Mice Are Protected Against Severe Blood Pressures in Salt-Sensitive Hypertension Study

For the majority of the protocol outlined in **Table 1** (weeks 2 through 7) the systolic blood pressure of the mice was determined using a tail blood pressure cuff ,with VEGF-D expression being induced from weeks 3 to 7. When plotted as a function of time, the data demonstrate that Kid-VD mice with increased lymphatic density in the kidney exhibit

significantly lower blood pressures under high salt challenge (**Figure 13**). As previously stated, the study would benefit from a time course experiment to determine whether lymphangiogenesis prior to, or concurrent with, the onset of hypertension is most beneficial.

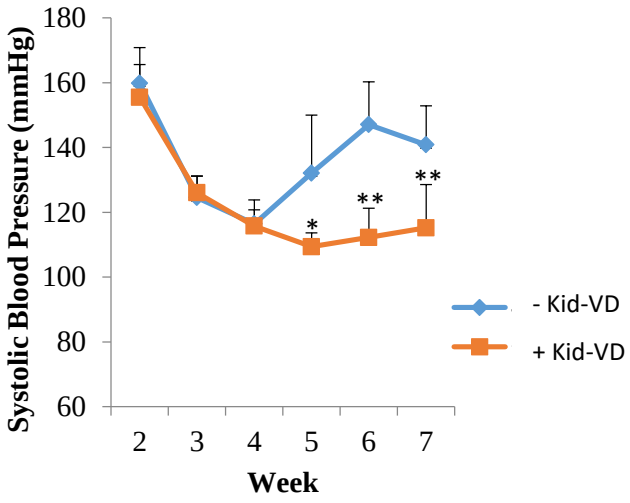

Figure 13. Systolic blood pressure measurements show protective effect conveyed by renal lymphatic hyperplasia. Measurements of systolic blood pressures of mice taken from week 2 of the protocol through week 7. The data show significantly decreased blood pressure measurements for mice having overexpression phenotype with development of salt-sensitive hypertension at week five.

qPCR Analysis of Inflammatory and Immune Cell Markers Shows Unresolved Inflammation

After demonstrating a protective effect regarding blood pressure, we sought to confirm the protective effect on a molecular level by comparing immune cell and inflammation markers. We hypothesized both of these groups to be lower in the Kid-VD mice than the mice lacking the genotypic modifications considering that both groups received the hypertension and inflammation challenges in the form of the high salt diet-induced salt sensitive hypertension.

IL1- β is an inflammatory marker, FGFR-1 is a fibrotic receptor, and increased levels of MCP-1 and CD11c indicate the presence of specific immune cell populations. As can be observed by the relative levels of IL1-β, MCP-1, CD11c, and FGFR1 gene expression in Kid-VD mice are all significantly elevated compared to the control mice (**Figure 14**). These elevated levels indicate that the protective effects provided by the expanded lymphatic network regarding blood pressure are not directly linked to a reduction in inflammatory and immune cell markers. Further studies need to be conducted to mechanistically determine the source of protection observed for systemic blood pressure and the cause for increased levels of immune cells and inflammatory markers.

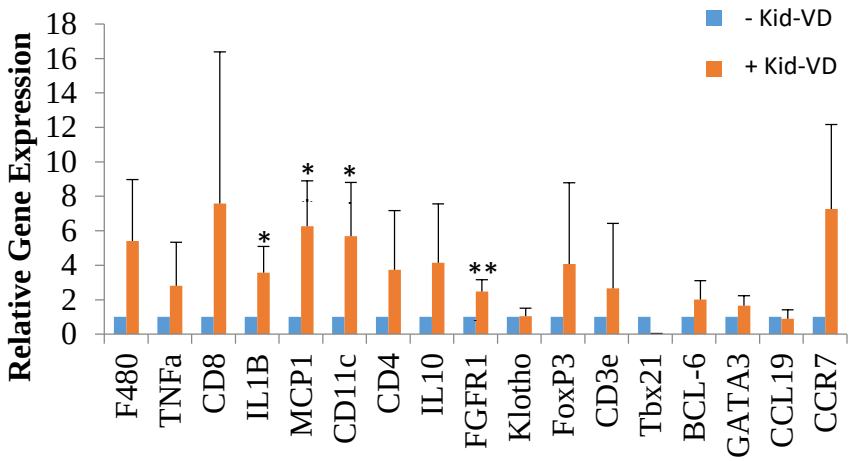

Figure 14. Positive VEGF-D mice show an elevation in inflammatory markers. qPCR analysis shows an elevation in inflammatory marker IL1-β, fibrotic receptor FGFR-1, and MCP-1 and CD11c bearing immune cells. Elevation indicates unresolved inflammation in the kidney compared to negative controls receiving the same dietary protocol. Additionally, chemokine CCL-21 (not depicted) showed a relative expression of 162.6 and p-value less than 0.05. (**p<0.01, *p<0.05).

CHAPTER IV

CONCLUSION

Throughout this study, mice with inducible lymphangiogenesis in the kidney were placed under different treatment protocols to investigate the effect of renal lymphatic hyperplasia on blood pressure regulation. The model used was that characterized by Lammoglia *et al*.[33] Rather than inducing lymphangiogenesis with a short treatment of concentrated doxycycline as described in the study, however, mice in this study were placed on 200 mg/L dox to grow a dense lymphatic vasculature over the course of 3-4 weeks.

In the L-NAME study, mice having renal lymphatic overexpression (along with controls) were treated with L-NAME, a nitric oxide inhibitor, to induce hypertension due to the stiffening and remodeling of the blood vasculature. qPCR analysis and histology confirmed the overexpression model and blood pressure measurements showed a potentially protective effect on systemic blood pressure. qPCR analysis of inflammatory and immune cell markers showed a slight elevation in immune cell subsets, but did not differ significantly.

In the salt-sensitive hypertension study, mice overexpressing renal VEGF-D (along with controls) were given L-NAME to cause vessel remodeling, resulting in salt-sensitivity. When placed on a 4% NaCl diet, these mice were subjected to salt-sensitive hypertension, which causes an inflammation challenge in addition to the blood pressure challenge. Similar to the L-NAME only study, qPCR analysis and histology confirmed the VEGF-D overexpression model. Unlike the previous study, however, blood pressure measurements were significantly lower in mice having overexpression of the renal lymphatics, suggesting a protective phenotype. qPCR

analysis, however showed significant increases in inflammatory and immune cell markers compared to the control mice.

Lymphatic vessels are responsible for maintaining fluid balance throughout the body. The primary mechanism by which this is done is by taking up the fluid extravastated from the blood vasculature once it has passed into the interstitium. In this manner, the lymphatics clear not only fluid, but also immune cells and cytokines found within the tissue. Consequently, the lymphatics also regulate inflammatory responses throughout the body.[40] In light of these know functions of the lymphatic system, we expected an expansion of the renal lymphatics to result in decreased populations of immune cells (such as CD4+ and CD8+ cells) and pro-inflammatory cytokines (such as TNFα) that are known to infiltrate the kidney in hypertensive states.[38] Our results show, however, that increased renal lymphangiogenesis is associated with increased immune cell and cytokine presence, bringing into question the functionality of the newly formed vessels. This is especially relevant considering that VEGF-D upregulation typically occurs only in states of inflammation. Overexpression in adipose, for example, results in increased infiltration of pro-inflammatory macrophages.[33] It is possible therefore, that the suggested increased immune cell presence is due to trafficking inefficiency rather than a mere "snapshot" of the immune cells present in the pathology. To determine whether this is the case, a study of interstitial fluid pressure manipulation effects in the presence of the expanded vasculature would prove beneficial.

In future studies, we plan to characterize the functionality of the expanded lymphatics, deduce the mechanism by which the renal lymphatics offer protection against systemic blood pressure increases, and determine the downstream effects of the infiltration of particular immune cell subsets into the kidney. We hope that, collectively, these inquiries will result in the

development of therapeutic targets for the resolution of hypertension in patients suffering from this ever-increasingly present cardiovascular disease.

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APPENDIX

Lymphangiogenesis: fuel, smoke, or extinguisher of inflammation's fire? Abouelkheir GR, Upchurch BD, Rutkowski JM. Exp Biol Med (Maywood). 2017 Apr;242(8):884-895. doi: 10.1177/1535370217697385

Lymphangiogenesis: fuel, smoke, or extinguisher of inflammation's fire?

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Impact statement

Inflammatory progression is present in acute and chronic tissue pathologies throughout the body. Lymphatic vessels play physiological roles relevant to all medical fields as important regulators of fluid balance, immune cell trafficking, and immune identity. Lymphangiogenesis is often concurrent with inflammation and can potentially aide or worsen disease progression. How new lymphatic vessels impact inflammation and by which mechanism is an important consideration in current and future clinical therapies targeting inflammation and/or vasculogenesis. This review identifies, across a range of tissue-specific pathologies, the current understanding of inflammation-associated lymphangiogenesis in the progression or resolution of inflammation.

Abstract

Lymphangiogenesis is a recognized hallmark of inflammatory processes in tissues and organs as diverse as the skin, heart, bowel, and airways. In clinical and animal models wherein the signaling processes of lymphangiogenesis are manipulated, most studies demonstrate that an expanded lymphatic vasculature is necessary for the resolution of inflammation. The fundamental roles that lymphatics play in fluid clearance and immune cell trafficking from the periphery make these results seemingly obvious as a mechanism of alleviating locally inflamed environments: the lymphatics are simply providing a drain. Depending on the tissue site, lymphangiogenic mechanism, or induction timeframe, however, evidence shows that inflammation-associated lymphangiogenesis (IAL) may worsen the pathology. Recent studies have identified lymphatic endothelial cells themselves to be local regulators of immune cell activity and its consequential phenotypes – a more active role in inflammation regulation than previously thought. Indeed, results focusing on the immunocentric roles of peripheral lymphatic function have revealed that the basic drainage task of lymphatic vessels is a complex balance of locally processed and transported antigens as well as interstitial cytokine and immune cell signaling: an interplay that likely defines the function of IAL. This review will summarize the latest findings on how IAL impacts a series of disease states in various tissues in both preclinical models and clinical studies. This discussion will serve to highlight some emerging areas of lymphatic research in an attempt to answer the question relevant to an array of scientists and clinicians of whether IAL helps to fuel or extinguish inflammation.

Keywords: Vascular endothelial growth factor receptor-3, lymphatic, vascular endothelial growth factor-D, metabolic syndrome, endometriosis, hypertension

Experimental Biology and Medicine 2017; 242: 884–895. DOI: 10.1177/1535370217697385

ISSN: 1535-3702 Experimental Biology and Medicine 2017; 242: 884–895

Introduction

The importance of the lymphatic vasculature and its fundamental roles have often been limited to the focus of narrow fields. For vascular biologists and tissue physiologists, lymphatics are part of the body's circulation.[1] For cancer researchers and clinicians, lymphatics are a route for metastatic cells to spread.[2] For immunologists, lymphatics carry immune cells, deliver antigens, and provide a site for immune cell interactions.[3] In each of these highlighted areas, lymphangiogenesis – the growth of new lymphatic vessels – is a potential mechanism for increasing that particular role or function. What has become increasingly appreciated, however, is that these roles are highly integrated and lymphatic "function" extends to all aspects of tissue homeostasis. The best examples of this integration are presented in instances of acute or chronic inflammation wherein lymphatics and lymphangiogenesis play a contextually dependent beneficial or detrimental role. To illustrate the importance of lymphatic biology and physiology, this review will identify how inflammation-associated lymphangiogenesis (IAL) extinguishes or propagates inflammatory progression in multiple tissues and diseases.

Lymphatic vessel and endothelial cell purpose

Lymphatic endothelial cell (LEC) biology and lymphangiogenesis have been extensively examined in a host of excellent reviews in the past decade (see the literature[4-7]). To understand how IAL specifically impacts the inflammatory progression, only a brief and basic summary of lymphatic roles and lymphatic biology is necessary. The smallest lymphatic vessels, lymphatic capillaries, reside in nearly all vascularized tissues as the low pressure sink to which extravasated fluid from blood vessels is removed from the interstitium.[1] This interstitial fluid, or what becomes primary lymph, contains all transportable macromolecules of the tissue. This cocktail is then intrinsically and extrinsically pumped along larger collecting lymphatic vessels to sentinel lymph nodes and is returned to blood circulation further downstream.[8] Disruption of the

lymphatics anywhere along this path by genetic manipulation or surgical resection results in peripheral lymphedema.[9] Consequently, fluid balance, and the resulting biomechanical and biochemical environment regulated by flow, is viewed as the predominant lymphatic role. Lymphatics also serve as a conduit for immune cell trafficking from the periphery by providing a route to the regional lymph nodes. The macromolecules transported via this path include cytokines, tissue fragments, hormones, and foreign antigens essential for both regular immune maintenance – tissue tolerance – and acquired immune responses.[3] The role of ‘‘fluid balance’’ is therefore more complex considering that disruption of flow impacts interstitial transport and that the composition of the fluid and the immune cells that traffic this route are both responsible for the classical lymphatic role in immune regulation. As a result, in instances of lymphatic malfunction such as lymphedema, many processes are disrupted: hydrostatic fluid balance is altered, cells do not experience interstitial flow, immune cells fail to properly traffic, and peripheral antigen transport is significantly delayed. The result: failure of both acute and chronic immune responses.[9–11] Recently, more active roles for LECs in immune regulation have been receiving more attention.[12–14] As was eloquently reviewed by Card et al.,[3] LECs have the ability to themselves take up and process antigens, alter immune cell phenotype and function, serve as a site of immune cell interaction, and secrete cytokines that propagate immune responses both locally and in the downstream node. These three roles of fluid balance, antigen and immune cell clearance, and immune response regulation are therefore not fully distinct. In reality, these functions are intimately linked and conceptually important in understanding how lymphatic vessels and the extent of IAL induce beneficence or harm.

Lymphangiogenesis regulation

Lymphangiogenesis is the development of the initial lymphatic vasculature, the growth of new vessels, and the hyperplasia of existing structures. Adult lymphangiogenesis is limited to acute

and chronic conditions of inflammation and tissue remodeling.[15] Adult lymphatic vessels are otherwise quiescent explaining the lack of a lymphatic vascular phenotype upon genetic ablation of the predominant lymphatic growth factor, vascular endothelial growth factor (VEGF)-C in adult mice.[16] The mechanism by which lymphangiogenesis occurs, most notably the cell source of new LECs, has recently been described to be much more complex than the traditional vascular sprouting described in blood angiogenesis and is very much tissue specific.[16–20] Multiple cell types may participate or assist in lymphangiogenesis as a source of lymphatic growth factors that induce LEC proliferation[6,15] and equally many antiproliferative cells and cytokines have been described.[15,21] The predominant and consistently key signaling mechanism for lymphangiogenesis is VEGF receptor (VEGFR)-3 activation by its ligands VEGF-C and VEGF-D.[4,7] LEC VEGFR-3 signaling is requisite in developmental and adult models of lymphangiogenesis.[5,22] Serum levels of VEGF-C and VEGF-D are elevated in inflammatory disease further confirming a driving role in IAL.[23–25] Focusing on VEGFR-3 signaling perhaps oversimplifies the mechanisms of IAL in which truly a host of pro- and antifactors are spatially and temporally at play; however, VEGFR-3 signaling is the gold standard for targeting lymphatic growth in preclinical studies and is now being manipulated in clinical approaches seeking to induce or resolve IAL.

Defining IAL

Lymphatic vessel roles make the system of increasing interest to the broader scientific community. Pathology slides are now routinely labeled for lymphatic protein markers LYVE1 or podoplanin by clinical and research labs alike and have identified changes to lymphatic size and density across a range of diseases. As previously mentioned, adult lymphangiogenesis is limited to states of acute or chronic tissue inflammation (or wound healing which we do not specifically

cover herein[26,27]). Most often, this inflammation and the consequences of the resulting IAL are dependent upon the tissue in which the remodeling is occurring. For this purpose, we accept IAL to be either expansion or generation of lymphatic capillary networks or the destabilization and hyperplasia of larger vessels. Thus, while lymphangiogenesis should increase fluid clearance, it is noted in multiple cases presented herein that IAL may merely result in expanded but less functional lymphatics. This review will highlight specific tissue pathologies in an attempt to give a broad overview of the contextually dependent beneficial and detrimental effects of IAL. Two well-studied IAL manifestations that are not necessarily limited to a particular region of the body, lymph node expansion and peritumoral lymphangiogenesis, are easily illustrated environments that provide an excellent basis to define potential roles of IAL and pathological lymphatic functions prior to discussing tissue-specific effects.

Inflammatory lymph node remodeling

Lymph node remodeling in response to afferent peripheral inflammation is a well-characterized process. Changes in the lymph node environment can include volume expansion from increased fluid burden, expansion of the LECstructured lymphatic sinuses, expansion of the fibroblastic reticular cell network, and proliferation of lymphocyte populations.[15] These changes are mechanically mediated and driven by vascular growth factors, cytokines, and CCL21-expressing T-cells entering with the afferent lymph as well as by lymph node reticular fibroblasts and B-cells (and later by neutrophils).[15] CCL21 is a noted LEC-secreted chemokine that guides leukocytes to lymphatic vessels for tissue egress; its levels are indicative in this review of both LEC numbers and activation.[5] Induction of proper lymph node expansion is a necessary part of resolving afferent inflammation and is documented in most IAL models in this review.[28] Once the peripheral inflammation is resolved, lymph node volumes recede and lymphatic structures

regress, a process driven at least in part by changes in the T-cell secretome.[29,30] Nodal expansion and subsequent resolution are thus a beneficial model by which to judge IAL.

Lessons from cancer research

Increased tumor-associated lymphatic vessel density and sentinel lymph node expansion correlate with higher incidences of metastases and poorer survival statistics in multiple cancer forms.[31–33] Lymphatic expansion by VEGF-C- or VEGF-D-expressing tumors is overall detrimental to patients, leading to dilation and hyperplasia of lymphatic vasculature,[2,34] increased lymph flow,[35,36] and increased lymphatic metastases.[2,37] One beneficial consequence of this process is that future therapies could potentially target the tumor spread via the upstream lymphatic network: a novel strategy of prevention and treatment of metastatic lymph nodes that shows promise in preclinical models.[38]

Tumor-associated lymphatics are, however, more than a metastatic highway. When tumors form, one component promoting their rapid progression is the ability to hijack immune signaling pathways.[39–41] This mimicry effect prohibits the host's innate immune response by activating more regulatory T-cells and preventing them from functioning against cancer cells, leading to tumor tolerance of the host's immune defenses.[39–41] Metastatic tumors are also known to secrete the leukocyte attractant CCL21,[42] a chemokine highly expressed by LECs, that serves to propagate leukocyte-driven lymphangiogenesis and essentially makes the tumor a rogue tertiary lymphoid organ (TLO).[43] The increased local LEC-immune interaction is essential for immunotolerance and the tumor's ability to evade normal immune responses.[43–46] Considering that the roles of lymphatics in tumor progression involve both local fluid and immune regulation, their effects serve as a model for understanding the impact of IAL on other disease states.

IAL in tissue inflammation pathologies

Immunohistochemical labeling has identified lymphatic vessels in most tissues – recent appreciation of functional lymphatics in the eye and brain only highlight the system's potential[47,48] – and a multitude of case reports identify increased or expanded lymphatic vessels with inflammation. For many tissues, a more complete characterization of lymphangiogenesis and, in most cases, direct evidence of the benefits or harm of IAL through the application of VEGFR-3 ligands (genetically, ectopically, or virally) or blockade of this pathway, is covered in multiple pathologies. In others, we cite literature wherein lymphatics themselves or lymphatic roles are referenced in the disease and extrapolate how changes in lymphatic density might impact tissue disease state.

Lymphangiogenesis in airway inflammation

Airway lymphatic vessels are necessary to regulate immune responses to the external environment and maintain fluid balance in the respiratory mucosa. Inhaled pathogens, including air pollution particles,[49] are transported via lymph to downstream lymph nodes for immune surveillance. Rodent models using Mycoplasma pulmonis infection have offered great insight into the mechanisms and function of IAL in the lung and airways.[50] Infection results in robust VEGFR-3 signaling and B- and T-lymphocyte-dependent lymphatic expansion that persists even after resolution of the infection.[51,52] While these lymphatics were morphologically different than native vessels (suggestive of altered function[53]), blocking IAL during infection results in increased mucosal edema.[51] Conversely, VEGF-C-induction of severe pulmonary lymphangiectasia, a condition of monstrous lymphatic expansion, also increased lung edema,[54] demonstrating that the quality of these newly developed vessels dictates their beneficence. Mycobacterium tuberculosis-induced granulomas also increase VEGF-C expression, resulting in local IAL.[55] Tuberculosis highlights active immune roles of LECs, with nodal IAL being

requisite for both antigen-specific T-cell responses[55] and, interestingly, the ability of M. tuberculosis to directly infect LECs for the purpose of proliferation.[56] In allergic rhinitis or asthma models, neither lymphangiogenesis nor vascular remodeling is consistently demonstrated.[57,58] Changes to lymphatic morphology have been reported in chronic obstructive pulmonary disease and could play a role in sustaining or priming inflammation, though no systematic study has yet been made of lymphatic changes.[59] What role bronchiolar lymphatic density plays in chronic asthma, obstructive pulmonary diseases, or how IAL impacts later responses to allergens or infection is not entirely clear.

Lymphangioleiomyomatosis (LAM) is systemic cystic disease but is often initially diagnosed through its pulmonary implications that include chylothorax (lymphatic, not hemal, fluid leakage). LAM pulmonary cysts are characterized by local IAL and massively elevated serum VEGF-D levels – 2- to 10-fold higher than healthy patients – may serve as a biomarker for the condition.[23,60] Human samples from other respiratory diseases such as pulmonary fibrosis and pneumonia samples have shown increased lymphatic density, lymphoid follicles, and elevated LEC-associated CCL21–CCR7 signaling.[61] Whether lymphatics serve as LAM sites and what role VEGF-D-mediated IAL plays in LAM and other lung pathologies remains to be elucidated.

Dermal inflammation and lymphangiogenesis

In the skin, IAL and changes to lymphatic patterning have been identified in both chronic[62] and acute[63] inflammatory skin disorders including psoriasis, atopic dermatitis, and systemic sclerosis.[62] Altered dermal lymphatics are also involved in dermal sodium balance[64] and tolerance sensitization.[11]

Acute dermal UVB-induced inflammation is characterized by enlarged lymphatic vessels and increased permeability leading to expanded inflammation and dermal edema.[63,65] Blocking

VEGFR-3, inhibiting lymphatic expansion, prolonged tissue swelling, and VEGF-C overexpression attenuated fluid accumulation through increased lymphatic vessel density.[63,66] While these models are well characterized, conditions such as atopic dermatitis and urticaria have also been correlated with vasculogenic factors, such as VEGF and semaphorins, that suggest a role for lymphangiogenesis in each disease.[62] In samples of human skin with psoriasis, the lymphatic vessels were enlarged,[67–69] VEGF-C was highly overexpressed, and skin mast cell numbers were elevated.[67–70] In acute mouse models, limiting IAL by inhibition of VEGFR-3 signaling increased inflammation and the infiltration, but presumably not the exit, of CD11bþ immune cells.[71] Conversely, overexpression of VEGF-C led to increased lymphangiogenesis and an overall reduction in chronic skin inflammation.[71,72] Consequently, in chronic skin disease models, lymphangiogenesis appears to inhibit or resolve inflammation.

Additionally, IAL has been implicated in contact hypersensitivity. K14-VEGFR-3-Ig transgenic mice lack dermal lymphatic vessels and have decreased solute transport and dendritic cell migration to the draining lymph nodes.[11] These mice showed greater swelling upon initial contact hypersensitization – demonstrating the importance of the lymphatic vessels in dermal fluid balance – but failed to tolerize to hypersensitization upon further challenge.[11] While these mice could elicit robust T-cell responses to dermal immunizations, the response was delayed and, with age, these mice developed autoantibodies to dermal proteins.[11] These studies demonstrate that healthy lymphatic transport is necessary for maintaining long-term immune tolerance.

Not only does lymphatic density play a role in inflammatory skin diseases, but they have also been implicated in homeostatic and blood pressure regulatory control systems in dermal cutaneous tissue.[64] Salt-sensitive individuals store excess electrolytes (Na+ and Cl-) in the skin interstitium that ultimately induce increased VEGF-C expression from infiltrating macrophages

and, consequently, lymphangiogenesis. In turn, the excess electrolytes are cleared by these new lymphatics.[64] Studies have also shown, however, that deletion of TonEBP (tonicity-responsive enhancer binding protein) from monocytes led to a lack of VEGF-C secretion, decreased clearance of electrolytes, and increased blood pressure.[64] Wiig et al. confirmed this effect by also inhibiting VEGFR-3, thereby decreasing cutaneous lymphatic vessel density and inducing hypertension by preventing sufficient clearance of excess electrolytes. VEGF-C overexpression had the opposite effect, increasing the uptake of electrolytes and water in the skin to further confirm the homeostatic role of dermal lymphatic remodeling.[64]

Lymphangiogenesis in intestinal inflammation

Gut lymphatics are not only responsible for surveying the massive bacterial load of the intestinal lumen, and the immunologic and inflammatory maintenance of such, but also play a lymphatic role that has not yet been touched upon: that of dietary lipid absorption in the intestine. Intestinal enterocytes take up digested and emulsified long chain dietary fatty acids from the lumen and repackage the fats into chylomicrons and very low-density lipoprotein particles that enter the initial lymphatic lacteal found within each villus. This chyle-rich lymph is transported through the submucosal lymphatics, large conducting mesenteric lymphatic vessels, and eventually on to the blood circulation at the thoracic duct. VEGFR-3-mediated deficiencies in the structure and maintenance of the lacteals disrupt lipid absorption, while changes in diet and hydration primarily affect mesenteric lymph flow rates.[73] Genetic and inflammatory alterations of lymphatic architecture result in lymph leakage.[74] Lymph leaked into the peritoneum is rich in soluble antigens and lipophilic bacterial products (e.g. lipopolysaccharide), and hence initiates the inflammatory immune cascade of macrophage and dendritic cell activation: a cycle of inflammation that likely further alters lymphatic function.[74,75] Lymph leaked in the mesentery is

also fatty acid-rich and highly adipogenic, causing expansion of the adipose tissue surrounding the collecting mesenteric

vessels.[74,76]

In chronic inflammatory bowel diseases from ulcerative colitis to Crohn's disease, lacteals dilate, submucosal edema occurs, and initial lymphatic capillaries proliferate.[73] It appears to be unlikely that IAL improves lymphatic drainage function under these conditions that manifest with rampant edema and failure of dendritic cells to migrate from the tissue,[77] but this may be partially caused by downstream reductions in pumping or lymphatic obstructions with lymphangitis.[73,78] In Crohn's mesenteric tissue biopsies, granulomatous lymphangitis further propagates inflammation as CD20þ B-cell rich TLOs reside along the mesenteric lymphatic vessels inhibiting flow and likely modulating the local immune response.[78] As in Crohn's, animal models of ileitis and colitis also exhibit lymphangiogenesis in the lymph nodes and lymphatic-associated TLOs, in addition to defective lymphatic drainage andpumping, as well as sustained mucosal edema and inflammation.[79,80] Expansion of the mesenteric adipose tissue, termed ''fat-wrapping'' or ''creepingfat,'' isa hallmark of Crohn's.Poorlymphaticfunction, accompanied by leaking mesenteric lymph and coupled with increased cytokine accumulation from the surrounding inflamed tissue, creates an adipogenic environment similar to that in well-characterized peripheral lymphedema models.[73,81,82] Conversely, mouse models with congenital intestinal lymphatic dysplasia or targeted deletion of LECs both exhibit increased intestinal inflammation.[21] Blockade of IAL using an antibody against VEGFR-3 has also led to increased leukocyte accumulation and edema in a spontaneous mouse irritable bowel disease model.[83] Destabilization of the existing lymphatic vasculature thus reduces the critical maintenance roles of gut lymphatics suggesting that IAL factors are overall detrimental to bowel diseases.

In addition to lymphatics being part of the body's greater circulatory loop, local lymphatics are critical in maintaining the health of the cardiovascular system. The lymphatic vasculature is particularly extensive within the heart and near the region of the vena cava, where lymphatic fluid is eventually returned to blood circulation.[1] Recently, myocardial infarctions (MI), an all-too-common cardiac pathology, have been identified as an inflammation-associated event with lymphatics playing a significant healing role. Post-MI inflammation is accompanied by increased VEGFR-3 signaling and lymphangiogenesis, particularly at the border of the scarred/infarct region.[17] Furthermore, VEGF-C administration after a myocardial infarct event induces lymphangiogenesis, improves cardiac function, and increases the drainage of the non-infarct region thereby improving prognosis.[17,84] Recently, delivery of adenoviral VEGF-D has also demonstrated improvement in cardiac function and is currently progressing to the clinic for further study. This work promisingly links cardiac lymphangiogenesis to improved patient health and future clinical

practice.[85,86]

Atherosclerosis is characterized by a buildup of cholesterol plaques in arterial walls.[87] Local lymphatic expansion (or lack thereof) and immune cell trafficking have been named key players in the inflammatory aspect of this disease.[88] One characteristic of the pathology is the conversion of macrophages to foam cells by the ingestion of lipids in the plaque and their inability to exit the tissue in the absence of lymphatic vessels.[89,90] Lymphatic vessels may also provide a route to clear cholesterol from the atherosclerotic lesion via reverse cholesterol transport (RCT). RCT is the process by which cholesterol is taken up by high-density lipoprotein (HDL) in the periphery and transported to the liver for processing and degradation.[91,92] Recent studies have implicated the lymphatics as the primary transport system by which peripheral HDL enters circulation.[91,93]

Lymphatic density may thus control the rate of tissue cholesterol clearance; several animal studies have demonstrated a disruption in RCT with a disruption of lymphatic vessels.[91,93] Lymphatics have also been implicated in venous health, with lymphatic ligation increasing venous lipid retention and TNF-alpha-mediated degeneration.[94] Martel et al.[93] also confirmed significantly impaired cholesterol removal from transplanted atherosclerotic aortas in which VEGFR-3 had been blocked. Mice with inherently reduced lymphatic density and impaired VEGFR-3 signaling also demonstrate increased atherosclerotic phenotypes.[95] It should be noted that the role of the lymphatics in this process is potentially one of local immune regulation or even active HDL transport from the tissue space by LECs themselves.[91] Collectively, the presence of lymphatic vessels and lymphangiogenesis appears to be beneficial in conditions of atherosclerosis. As such, increasing lymphangiogenesis at an atherosclerotic site could prove to be therapeutic in the clinic.

Considering their close association with arterial walls, it is reasonable to ponder whether lymphatics play a role in vascular inflammation, venous disease, and hypertension. We have already discussed how dermal lymphatics play a role in sodium storage and, hence, potentially salt-sensitive hypertension.[96] In other organ systems, lymphatic function may locally regulate endocrine tissue homeostasis and their hormone secretion and transport, such as the renin–angiotensin–aldosterone system present in the kidney.[97] Recent work in the kidney, for example, has implicated changes in renal T-cell populations and inflammation with hypertension in both human samples and animal models.[98] With the lymphatics regulating immune phenotypes, tissue inflammation, cholesterol transport, and salt retention, lymphangiogenesis potentially plays complex and multitudinous roles in regulating cardiovascular health.

The field of renal health and kidney inflammation more specifically is associated with conditions such as hypertension, diabetes, and proteinuria.[99] Little is published about the physiological functions of renal lymphatics, and less so in these disease states. In animal models, ligation of renal lymphatics results in renal edema, increased urine volume, and hypertension.[100,101] Two case reports describing patients presenting with hypertension have diagnosed concurrent renal lymphangiectasia. In these cases, hypertension is attributed to the mechanical stress applied by hyperplastic collecting lymphatic vessels.[102,103] In one case study, the hypertension was resolved upon removal of the collected fluid, while in another the patient's hypertension was resolved with angiotensin-converting enzyme inhibitors, pain medication, and salt restriction.[102,103] Lymphatic function in immune cell trafficking may present a clearer role: increased T-cell infiltration is necessary for development of Dahl salt-sensitive hypertension via aberrant activation of the renin–angiotensin system in rats.[104–106] While inflammatory immune cells are a source of VEGFR-3 ligands, none of these studies directly implicate IAL, or a lack thereof, to hypertension specifically. We recently presented a model of VEGF-D-driven expansion of the renal lymphatic vasculature (Figure 1) that could potentially be used to assess how lymphatic function impacts blood pressure in a variety of hypertensive models.[107] Based on the results in other tissues, inducing renal lymphangiogenesis may alleviate the kidney's inflammatory burden and increase immune cell egress in these models.

Renal diseases often manifest with concurrent inflammation, fibrosis, and proteinuria. Proteinuria is not only a symptom but also directly damages tubular epithelia resulting in pro-inflammatory CC- and CXC-chemokine secretions.[108] These chemokines also promote the recruitment of circulating leukocytes, such as regulatory T-cells, and therefore, indirectly promote IAL.[109] Lymphangiogenesis has also been previously correlated with renal fibrosis in

several studies.[110–113] Renal lymphangiogenesis has been identified, however, prior to the development of marked fibrosis.[114] Blocking CCL21, a LEC-secreted chemokine, reduces kidney fibrosis by preventing the infiltration of fibrocytes and macrophages.[115] Together, these evidences suggest IAL occurs early with proteinuria and is potentially necessary, or merely an early indicator of, the development of subsequent fibrotic phenotypes. In contrast to other IAL events, lymphangiogenesis could be targeted to prevent proteinuria-mediated tubulointerstitial fibrosis; however, chronic lymphatic ligation worsens renal inflammation, fibrosis, and proteinuria[100] so disruption of homeostatic lymphatic function may have severe consequences.

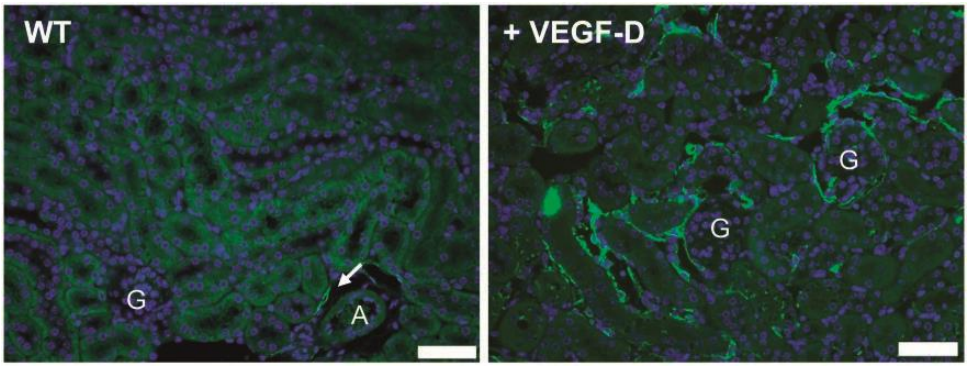

Figure 1 Expansion of the renal lymphatic network. Lymphatic vessels are few in the murine renal cortex limited to tracking along interlobular arterioles (arrow, left). Overexpression of VEGF-D (right) by tubular epithelial cells induces a massive expansion of cortical lymphatic vessels (green, LYVE-1) providing a model to study the impact of lymphatic density in renal function and pathology. ''A'' indicates arteriole. ''G'' indicate glomeruli. Blue ¼ DAPI. Scale bars ¼ 50 mm. VEGF-D: vascular endothelial growth factor-D

In healthy kidneys lacking any sort of pathology, relatively few lymphatic capillaries are present within the cortex of the kidney.[99] Despite their relative scarcity, their chronic ligation is severely detrimental to renal function and inflammation.[100] Chronic inflammatory conditions such as lupus nephritis, antineutrophil cytoplasmic antibody-related glomerulonephritis, tubulointerstitial nephritis, and IgA nephropathy show markedly increased populations of lymphatic vessels in the renal cortex compared to controls.[111] Additionally, Type 2 diabetic nephropathy biopsies have shown both increased VEGF-C expression and elevated lymphatic

density.[111] A study focusing specifically on tubulointerstitial nephritis found that the number of lymphatic vessels was significantly correlated with the degree of fibrosis and that infiltrating monocytes were expressing VEGF-C.[111] Biopsies taken from chronic interstitial nephritis or chronic IgA nephropathy patients showed significantly elevated lymphatic density compared to biopsies of acute tubulointerstitial nephropathy patients.[116] These results suggest that sustained inflammation must be present for a substantial period of time prior to (or to initiate) lymphangiogenesis. Whether lymphatic expansion is actively involved or merely a marker of these pathologies, however, remains to be determined, though it is intriguing to speculate that by having fewer lymphatics when healthy, the kidney is ripe for IAL causing dysfunction.

Adipose tissue, the metabolic syndrome, and lymphatic function

Systemic manifestations of the metabolic syndrome – hyperlipidemia, hyperglycemia, and insulin resistance – all have their root in dysfunctional obese adipose tissue that is characterized by inadequate vascularization and increased fibrosis and inflammation.[117] In mouse models targeting inflammation, either by reducing immune cell populations in adipose tissue or by genetic manipulation of inflammatory cytokines, systemic metabolism is improved.[118] What role lymphatic vessels play in regulating the adipose tissue interstitium is still unknown in normal physiology, but feedback between the lymphatics and adipocytes is clear. Adipose tissue hormones, collectively adipokines, have potent effects on endothelial cell biology.[76] Adiponectin, for example, increases nitric oxide synthase in LECs and adiponectin treatment aides in ameliorating lymphedema.[119] How other adipokines, such as leptin, impact LEC biology and function is also of timely interest in many research groups.[120] Multiple mouse models targeting lymphatic vessel development and maturation demonstrate increased adiposity accompanied by adult onset obesity.[76] In lymphedema, when lymph flow is compromised, potentially massive

adipose expansion occurs in the affected periphery.[121] In inflammation, leaky lymphatics driven by IAL factors are commiserate with expansion of the adipose directly around the destabilized lymphatic vessel. Inflammation models have also demonstrated local and lymph node adiposity with IAL.[73] Adipose–lymphatic interactions are not all malicious, however, as perinodal adipose tissue and adipose-resident monocytes along collecting lymphatic vessels play an important role in maintaining lymph node and immune surveillance functions,
respectively.[73,75]

Measurements of lymph flow in generalized obesity demonstrate reduced lymphatic function, and its improvement with weight loss and exercise in the same animals.[122,123] In lipedema, a condition of pathologic adipogenesis, lymphatic function is reduced and potentially plays a role in disease progression.[15] A multitude of adipokines and growth factors are expressed in healthy and obese adipose and their relative distributions may impact local lymphatic integrity and function as well as IAL.[76] VEGFC and VEGF-D are heightened in obesity; a recent study of systemic VEGFR-3 blockade improved glucose regulation in part through reduced adipose tissue macrophage infiltration.[24] Similarly, mice with constitutive dermal overexpression of VEGF-C exhibit hyperplastic lymphatic vessels, heightened adipose inflammation, and increased adiposity and insulin resistance.[124] Recently, we developed a mouse model with inducible adipocyte-specific overexpression of VEGF-D and, while the mice demonstrated a massive expansion of lymphatic networks in adipose tissues, macrophage recruitment and inflammation were also increased.[107] With high fat diet feeding that normally drives adipose tissue inflammation, fewer resident macrophages were found with increased lymphatic density (Figure 2). Uncoupling the negative chemokine aspects of these lymphatic growth factors from the potentially positive effects of increased lymphatic density is currently underway.

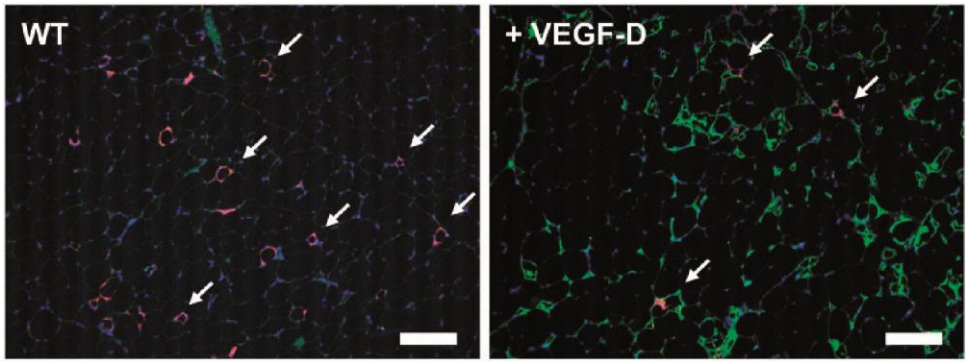

Figure 2 Adipose tissue lymphangiogenesis. Obese murine inguinal adipose tissue lacks lymphatic capillaries (green, LYVE1) and contains macrophages formed crown-like structures (red, Mac2; arrows highlight some) in the inflamed tissue (left). VEGF-D overexpression induces lymphangiogenesis in adipose tissue (right), with IAL potentially providing a route for immune efflux with fewer Mac2þ areas (arrows, all noted) in equally obese adipose tissue. Blue ¼ DAPI. Scale bars ¼ 100 mm. VEGF-D: vascular endothelial growth factor-D

Though adipose inflammation may be a target for lymphangiogenesis in the metabolic syndrome, diabetic lymphatic function, or, perhaps more importantly, direct lymphangiogenic effects on beta cells' insulin secretion and the pancreatic islets is less clear. Islets have intra-islet blood capillaries, but lymphatic capillaries are only found in the islet periphery. In two models in which VEGF-C or VEGF-D was directly overexpressed in beta cells, no lymphatic ingrowth to the islets was observed, despite marked extra-islet lymphatic expansion.[34,125] In the commonly used model of streptozotocin (STZ)-induced islet toxicity, IAL was identified around the islets.[126] Inhibiting IAL in the STZ model by blocking VEGFR-3 reduced islet cytokine levels as well as macrophage infiltration and preserved islet mass (and, by extension, insulin secretion).[126] Whether islet IAL is good or bad may therefore depend on the underlying mechanism of beta cell loss, i.e. Type 1 versus Type 2 diabetes. No large-scale systematic study in humans has yet identified peripheral lymphatics to be functionally altered in diabetes, but in some patient samples and murine models, insulin resistance and obesity, not high fat diet alone, reduces lymphatic fluid transport.[127,128] Since weight loss and exercise alone each rapidly improve obesity-associated lymphatic dysfunction,[123,127] are lymphatic deficiencies merely reflective of greater tissue imbalance? The importance of lymphangiogenesis and LECs in modulating

peripheral inflammation, however, still makes lymphatic expansion an attractive potential target in dysfunctional adipose tissue in obesity, lipedema, and lymphedema and in the peripheral vascular manifestations of diabetes.

IAL in female reproductive tissues

Lymphangiogenesis occurs in the ovaries and uterus with reproductive cycles and pregnancy[26,129]; however, inflammatory diseases of the female reproductive tissues are largely understudied and few murine models exist to recapitulate them. Endometriosis is a disease uniquely affecting women in which tissue resembling the uterine lining – the endometrium – is found in tissue outside the uterus, as far away as the lungs.[130] One explanation is similar to the theory of LAM lesion spreading: that shed endometrial tissue and cells are transported via the lymphatic system to other regions of the body causing lesions at ectopic sites.[131] Reichelt et al.[132] demonstrated an increase in the lymphatic density of peritoneal lesions compared to normal peritoneum. In one study, VEGF-C and VEGF-D were both found to be significantly upregulated in endometriotic lesions and accompanied by significant lymphangiogenesis (and minimal blood angiogenesis) in ectopic lesions.[132] Endometriosis is at least partially a chronic inflammatory disease, in which the inflammation is perpetuated, rather than resolved, by the lymphangiogenesis.[132] Further work beyond male mouse models may identify what roles lymphatics play in endometriosis and other diseases of women's health.

Transplantation neovascularization

One area where the role of lymphatics is often emphasized is in the context of tissue transplants. Transplant outcomes demonstrate the range of responses associated with IAL as often a mixture of harm and benefit depending on the context. In cardiac allografts, most often, lymphangiogenesis increases the chances of rejection, with blockage of VEGFR-3 signaling improving the outcome of the allograft.[133] This result is likely due to the decrease in CCL21

expression and CD8þ T-cell (lymphocytes that regulate tolerance) infiltration upon blocking VEGFR-3.[133] Another common transplant highlighting a potentially negative role of the lymphatics is that of corneal transplants. In instances of both human and animal keteroplasties, corneal lymphangiogenesis decreases the graft's survival and is an indication of impending rejection.[134–137] Several studies show that antivascular treatment both pre- and postprocedure improve the chances of corneal graft survival.[136,138,139]

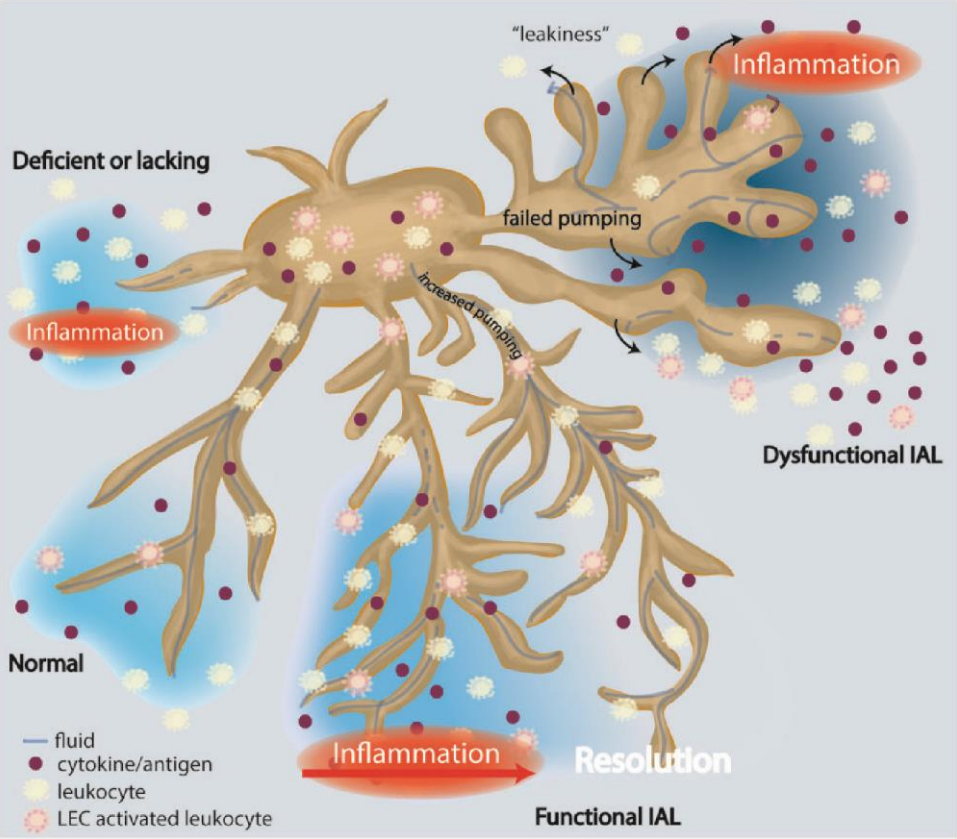

Figure 3 Inflammation-associated lymphangiogenesis (IAL) and the modulation of tissue homeostasis. Normal, functional lymphatics clear fluid, macromolecules, and immune cells – both passively and actively – from the peripheral interstitium. Trafficking to the afferent lymph node allows for the propagation of specific immune responses. When lymphatics are deficient (left), as in lymphedema, failed clearance leads to chronic inflammation. With IAL in inflammation, an increased functional lymphatic network permits resolution as fluid and cytokines are cleared and leukocytes – activated locally by inflammation or through contact with lymphatic endothelial cells (LEC) – traffic to the lymph node. Disorganized lymphatic expansion in inflammation, however, results in lymph efflux, or ''leakiness'' of hyperplastic vessel (right).This reduced functional capacity fails to aid, and indeed can propagate, inflammation

The effect of IAL in renal transplants, however, is more ambiguous. In the three weeks following kidney transplant, the donor organ generates a new lymphatic system.[140] This process

is crucial, and low lymphatic density in the first postprocedural biopsy has been correlated with acute transplant rejection.[141] This is likely due to the role of the lymphatics in fluid clearance, inflammation modulation, and immune cell clearance after the operation.[142] Once the initial IAL takes place, however, persistent lymphangiogenesis and increased lymphatic density in regions of inflammation have been associated with transplant rejection.[141] Whether the continuing lymphangiogenesis is merely a marker or a cause of rejection is currently unknown. One possible explanation is that, after achieving homeostatic fluid balance, the increased presence of lymphatics allows antigenpresenting cells to induce an allogenic response as in the cornea.[142] As more is understood about the evolution and functions of these vessels following transplant, however, more specific therapies can be designed to improve the chances of allograft survival.

Conclusion

No region of the body is impenetrable to the flames of inflammation. The body's natural response to tissue injury is inflammation, often accompanied with lymphangiogenesis, that is a required part of healing. This IAL and its associated responses, much like fire itself, can be classified into a few broad categories: absent, functional, and dysfunctional (Figure 3). In instances when IAL results in the development of functional vasculature, it serves its purpose of fluid and macromolecule reclamation and immune cell modulation and clearance resulting in restoration to a preinflammatory state. In this way, IAL performs its physiological duties similar to a beneficial fire that is extinguished when needed. Much like a wildfire, however, left to progress unchecked, IAL results in poorly functional vessels, a worsened immunologic state, and unresolved inflammation. As a result, the simple question of whether IAL is beneficial or harmful receives the unsatisfying answer of: it depends. Lymphatics are at the center in the interplay of fluid clearance, inflammatory cytokine removal, and innate and acquired immune regulation. Understanding IAL and its degree of beneficence on a disease- and tissue-basis is

requisite in determining whether lymphangiogenesis will prove to be a valid therapeutic target for inflammation resolution and the treatment of the myriad of diseases plaguing today's patient populations.

Authors' contributions: All authors made substantial contributions to the literature research, writing, and editing of the manuscript.

ACKNOWLEDGEMENTS

JMR is supported in part by the American Heart Association (12SDG12050287) and a research grant from the University of Pennsylvania Orphan Disease Center (MDBR-16-118-GLA/ GSD).

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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